

**Conserving amphibian diversity: a  
species inventory and gene flow studies  
in fragmented montane forest,  
Mambilla Plateau, Nigeria**

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A thesis submitted in partial fulfilment of the requirements

for the Degree of Doctor of Philosophy in Ecology

at the University of Canterbury

by

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University of Canterbury

February 2015

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# Acknowledgements

I am deeply grateful to my supervisors Professor Hazel Chapman, Dr. David Blackburn (California Academy of Sciences), especially to Dr. Marie Hale for her patience and infinite support.

I would like to thank to each and all of the field assistants and staff from the Nigerian Montane Forest Project. I am extremely grateful to Thomas Patrick and Muhammed Adam (Hamadu) for their positive attitude even during the rainiest night in Kurmin Danko. Without them my field work would not have been as amusing and productive.

During the lab work -likely the hardest but also the most rewarding part of this PhD- I had the unconditional assistance of Maggie Tisch and Craig Galilee to whom I am very grateful. Also, helping me in the lab were Thomas Evans and Claire Galilee.

I would like to thank to Jenny Ladley for her support when preparing my field work, and Matt Walters for his amazing ideas when putting together posters and presentations related to my PhD project. Huge thanks to Matt Wallace for helping me in the first stage of my lab work, and to all members of the molecular lab group for their valuable feedback, especially to Tammy Steeves whom I admire due to her honesty.

All my gratitude goes to my Mom and Dad who have given me their unconditional love and support ever since I decided to go to New Zealand to complete, so far, the greatest challenge of my life. Thank you to my brother for his helpful and equable advice, and special thanks to my sister who despite the distance was always with me giving me words of encouragement to keep going during the difficult stages of the development of this PhD.

I would like to thank my friends Lupe, Carol, Juliano, Nick, and Leighton for their friendship and support during a gloomy time when I was not in good health. I would also like to thank my lab friends Rachel, Jasmin, Ilina, Ayla, and Natalie, for all the fun moments in the lab, and for those special Sundays of cooking delicious international dishes.

Finally, I am grateful to my sponsor the Consejo Nacional de Ciencia y Tecnología – CONACYT (the Mexican National Council for Science and Thechnology) for its financial support during my PhD studies.

# **Abstract**

Nigeria is the most densely populated country in Africa and one of the most advanced economically in terms of both industry and soil and landscape utilization. This country is projected to have one of the largest urban growth rates by 2050. Thus, the demands of the rapidly increasing human population and its material consumption represent a severe threat to biodiversity. Nigeria has the highest deforestation rate of natural forest in the world, its original vegetation has largely been replaced by farming activities, urban development and other products of human activities. The principal causes of the decline and loss of biodiversity in Nigeria include human exploitation of natural resources, fragmentation of habitats and populations, conversion of wild areas to agriculture and other intensive human use and alterations in the structure and function of ecosystems. Amphibians are the vertebrate group with the highest number of species threatened with extinction and habitat loss and fragmentation are considered to be among the leading causes of their declines and extinctions. It has been recognized that one of the most severe problems in conservation biology is the scarcity of baseline data. Such lack prevents evaluation of the effect of the expanding anthropogenic impact and determination of potential population declines.

The mountains of eastern Nigeria, within Taraba State, are regionally important in terms of biodiversity and endemism, however, its herpetofaunal diversity has received little attention. Moreover, no studies have investigated how habitat loss and fragmentation may affect dispersal and gene flow among small and isolated amphibian populations, and in the absence of such studies attempts at amphibian conservation are compromised.



The aims of this project were threefold. Firstly, a comprehensive inventory of the amphibians and reptiles of Ngel Nyaki and Kurmin Danko Reserve on the Mambilla Plateau was compiled. The outcome, an annotated list of 21 amphibians and 11 reptiles, represent the most thorough inventory to date of the herpetofauna on the Mambilla Plateau. Based on this inventory four key anuran species were selected to conduct a population genetics study.

Secondly, molecular tools specifically AFLP markers were developed and used to analyze the genetic population structures of the four frog species *Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1 and *Astylosternus* sp. 2. differing in geographic distribution and life history traits within the study area.

Thirdly, these species were assessed to understand dispersal and connectivity among fragmented and continuous populations on the Ngel Nyaki and Kurmin Danko Reserve. Genetic differentiation among the forest and the riparian fragment populations was observed for three of the target species, however, no significant genetic differentiation was detected among the populations located in continuous forest for any of the four frog species. In addition, geographic and genetic distances were not correlated significantly for any of the four target species, suggesting no isolation by distance at this fine geographic scale.

Results from both the inventory and the genetic population structure study revealed that the riparian forest fragments are of utmost importance for the persistence and migration of *Cardioglossa schioetzi*, and potentially for many other amphibian species. The new scientific findings are now part of the valuable baseline data on the diversity and genetic population structure of amphibian species in Ngel Nyaki and Kurmin Danko Forest

Reserve. These results will better inform conservation managers who need to make decisions around management of montane habitat for amphibian species.

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# Chapter 1

## General Introduction

### 1.1 The problem

Nigeria is the most densely populated country in Africa and one of the most advanced economically in terms of both industry and soil and landscape utilization (Singh *et al.* 1995). According to the Population Division of the Department of Economic and Social Affairs, United Nations, the 2014 revision of the World Urbanization Prospects indicated that by 2050 Nigeria is projected to add 212 million urban inhabitants, then along with India and China, it will have one of the largest urban growth rates (United Nations [UN], 2015). The demands of the rapidly increasing human population and its material consumption represent a severe threat to biodiversity (Primack 2012). Nigeria's original vegetation has largely been replaced by farming activities, urban development and other products of human activities (Akinpelu and Areo 2007). In 2005 Nigeria was reported to have lost 55.7% of its primary forest to logging, subsistence agriculture, collection of fuelwood, and other agents (Olanrewaju 2008). Deforestation is a serious problem in this country, with forest loss occurring at a rate of 3.3% per year (Orimoogunje *et al.* 2009). These figures give Nigeria the highest deforestation rate of natural forest in the world (Butler 2005, Babanyara *et al.* 2010). Thus, human exploitation of natural resources, fragmentation of habitats and populations, conversion of wild areas to agriculture and other intensive human use and alterations in the

structure and function of ecosystems are the principal causes of the decline and loss of biodiversity in Nigeria (Aremu *et al.* 2009).

Amphibians are the vertebrate group with the highest number of species threatened with extinction and habitat loss and fragmentation are considered to be among the leading causes of their declines and extinctions (Blaustein *et al.* 1994, Blaustein and Wake 1995). Other factors include infectious disease (Berger *et al.* 1998, Catenazzi *et al.* 2011), climate change (Lowe 2012) and the introduction of exotic species (Escoriza and Boix 2012), while unknown factors also contribute (Houlahan *et al.* 2000, Stuart *et al.* 2004, Lips *et al.* 2005). According to the IUCN Red List (IUCN 2015) 36 amphibian species are extinct and 1957 are threatened, that is, listed as Vulnerable, Endangered, and Critically Endangered.

## **1.2 Value of species inventories**

It has been recognized that one of the most severe problems in conservation biology is the scarcity of baseline data; the scarcity of knowledge about the diversity for some regions makes it extremely difficult to protect their threatened species (Stuart *et al.* 2008). For example the absence of distribution and ecological data for many amphibian species makes it extremely difficult to determine whether populations are declining or resisting the trend of global decline (Stuart *et al.* 2004). The value of compiling quantitative baseline data for amphibian fauna has been highlighted by several researchers (e.g. Bury *et al.* 1980, Busby and Parmelee 1996, Joger and Lambert 1996), particularly for biodiversity hotspots (Herrmann *et al.* 2005). Species inventories in areas which have not yet been studied are crucial because they may result in discovery of new species and extend current range distributions of other species. Moreover they provide information on habitat and population status (Joger and Lambert 1996). As



pointed out by Joger and Lambert (2002), herpetofaunal inventories provide not only documentation of species distribution, but also help to identify centers of richness and endemism.

While the diversity of amphibians and reptiles is relatively well documented in many tropical regions (Duellman 1999, Lips *et al.* 2005), data from Central Africa is poorly known or remains entirely unknown. Such is the case for Nigeria where some areas have received little attention in terms of amphibian diversity (Bergl *et al.* 2007). This is especially true for the mountains of eastern Nigeria, part of the Cameroon Volcanic Line (Marzoli *et al.* 2000) where taxonomic and distributional studies of amphibian fauna are limited (Schiøtz 1963, Böhme and Nikolaus 1989, Blackburn 2010, Blackburn *et al.* 2010, Blackburn and Rödel 2011).

The Cameroon Volcanic Line, whose geological structure consists of the mountains of Nigeria and Cameroon and the Gulf of Guinea Islands (Marzoli *et al.* 2000) is perhaps one of the most biologically diverse areas of the African continent (Burgess *et al.* 2004). The mountains of eastern Nigeria, within Taraba State, are regionally important in terms of biodiversity and endemism (Chapman and Chapman 2001). Of all African habitats, montane forests are among the most susceptible to degradation and loss (Dunn 1999) and the montane forests of Taraba State are threatened by expanding agricultural activities, illegal logging, human settlements and overgrazing by cattle. The resulting habitat degradation and loss are likely important drivers of amphibian population declines in this poorly known region (Stuart *et al.* 2004). Previous surveys conducted as part of a multidisciplinary expedition to the Gotel Mountains and the Mambilla Plateau in eastern Nigeria in 1989 recorded the frog species *Astylosternus* cf. *ranoides*, *Cardioglossa* sp., *Leptodactylodon* sp., *Phrynobatrachus* cf. *werneri*, and *Phrynobatrachus* sp. in Gangirwal, Gotel Mountains but only two species,

*Amietophrynus maculatus* and *Leptopelis nordequeatorialis* from the Ngel Nyaki Forest Reserve (Figure 1-1) (Böhme and Nikolaus 1989), the main study site of this thesis. Twenty years later, a rapid survey of Ngel Nyaki and neighbouring Kurmin Danko forests in 2009 resulted in discoveries of two new frog species, *Arthroleptis palava* (Blackburn *et al.* 2010) and *Phrynobatrachus danko* (Blackburn 2010), the latter of which is so far known only from Kurmin Danko. Following these discoveries a comprehensive inventory of the amphibians and reptiles of Ngel Nyaki and Kurmin Danko Reserve was compiled as part of the current study, and is presented in chapter 2.



Figure 1-1. Localization of Gangirwal in the Gotel Mountains and Ngel Nyaki in the Mambilla Plateau. Reproduced from Chapman and Chapman 2001.

### 1.3 Population genetics

Amphibian conservation efforts can be greatly aided by an understanding of population structure and of the ability of target species to disperse (Beebee 2005, Beebee and Griffiths 2005). No studies have investigated how habitat loss and fragmentation may

affect dispersal and gene flow among small and isolated amphibian populations on the Mambilla Plateau, and in the absence of such studies attempts at amphibian conservation will be compromised. An understanding of dispersal abilities and how populations respond to landscape change are essential for successful conservation and management plans (Gibbs 1998, Bowne and Bowers 2004). For instance, redressing fragmentation effects would depend upon a better understanding on movement of individuals among habitat patches (Bowne and Bowers 2004). To address this lack of knowledge, an important aim of this thesis was to select key anuran species for a population genetics study. In chapters 3 and 4, molecular tools are developed and used to analyze the genetic population structures of four frog species differing in geographic distribution and life history traits within the study area: *Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1 and *Astylosternus* sp. 2. These species were assessed to understand dispersal and connectivity among fragmented and continuous populations on the Ngel Nyaki and Kurmin Danko Reserve. While the detection of genetic effects is critical for the proper management and persistence of fragmented populations, an understanding of patterns of genetic structure in continuous habitats is also essential for determining how these patterns are altered by fragmentation (Cabe *et al.* 2007).

Habitat fragmentation alters population demography through decrease in habitat availability that in turn increases isolation of each remaining habitat fragment; consequently, the reduced habitat area impacts the viability of local populations by reducing population size, and the isolation reduces immigration from other populations (Reviewed in Joly *et al.* 2003). Likewise, fragmentation of habitat has severe genetic implications. A population that has been fragmented might face genetic changes such as differentiated populations and loss of variation among individuals from the same

population (Cushman 2006). Small and isolated populations usually lose genetic variation more quickly than larger and continuous populations due to genetic drift (Lacy 1987), and might undergo decreased fitness because of an increase in the expression of deleterious alleles, inbreeding depression, and loss of adaptive potential (Frankham *et al.* 2002).

Amphibians are likely to be severely affected by habitat fragmentation because many species have poor dispersal abilities (Beebee 2005), are integral components of terrestrial and aquatic environments (Semlitsch 2003), present relatively narrow moisture and temperature tolerances (Duellman and Trueb 1986, Rittenhouse *et al.* 2008), and have specific habitat requirements for their reproduction and life history (Semlitsch 2003). Moreover, species with a high degree of ecological specialization and restricted dispersal are more susceptible to habitat deterioration (Chan and Zamudio 2009).

In order to understand genetic differentiation over a geographic range, estimates of gene flow are needed (Berven and Grudzien 1990). Because the geographic limits of the neighborhood into which organisms can be grouped are based on dispersal distance, dispersal ability is essential to the genetic population structure of organisms (Berven and Grudzien 1990). Dispersal is one of the most significant biological processes shaping the distribution and abundance of all animal, plants, and other organisms (Lindenmayer and Fischer 2006). Dispersal might depend on the interaction between the dispersal behavior of a given species and the physical attributes of the landscape wherein movement is performed (Calabrese and Fagan 2004, Baguette and Van Dyck 2007).

Estimates of dispersal derived from genetic tools represent a suitable alternative to direct methods such as mark-recapture that require a considerable investment of field

work and may underestimate gene flow (Slatkin 1987). Thus, genetic tools that allow reliable estimates of gene flow are effective for evaluating potential barriers to gene flow (e.g. Lampert *et al.* 2003, Zamudio and Wiczorek 2007, Baguette *et al.* 2013).

The use of molecular markers to reveal patterns of genetic structure provides an indirect way to infer dispersal rates and patterns of recent and historical gene flow among populations (Mila *et al.* 2010). Currently there are many molecular marker techniques available; the most widely used are Random Amplified Polymorphic (RAPD) (Williams *et al.* 1990), Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al.* 1980), Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.* 1995), and microsatellites or Simple Sequence Repeats (SSR) (Tautz 1989). These markers differ from each other in terms of the amount of variability expressed, ease and costs of their development and value in answering particular questions.

One of the most popular genetic markers used in conservation genetics are microsatellites (Abdelkrim *et al.* 2009). Microsatellites are appropriate for the study of fine grain population structure (Zamudio and Wiczorek 2007, Austin *et al.* 2011) because of their high mutation rates and simple Mendelian mode of inheritance (Selkoe and Toonen 2006, Abdelkrim *et al.* 2009). In spite of their utility in population genetics, there is a lack of species-specific markers for many groups, particularly for African amphibians. Species-specific microsatellite markers have been developed for only two African amphibian species. Sandberger *et al.* (2010) isolated four polymorphic microsatellites for the frog species *Phrynobatrachus guineensis* (Phrynobatrachidae), which were the first microsatellites described for any African amphibian species. Barratt *et al.* (2012) later isolated nine polymorphic microsatellite markers for the caecilian *Boulengerula cf. uluguruensis*. No species-specific microsatellites were available for any of the target species in this study, nor for any species within the large radiation of

frog species in the Afrobatrachia, including the species in the Arthroleptidae studied here. The development of species specific primers is costly and time consuming (Abdelkrim *et al.* 2009). Combined with the urgency to rapidly establish conservation priorities for the target species, this lack of primers led to the development of AFLPs for the assessment of genetic population structure in this study. These markers are the most suitable alternative when no specific microsatellites are available for a given species (Gaudeul *et al.* 2004, Measey *et al.* 2007). Even in some cases when microsatellites are available, AFLPs can provide better resolution (Mueller and Wolfenbarger 1999, Bensch and Åkesson 2005). Some of the advantages of AFLPs over other molecular markers include the fact that no prior DNA sequence information is necessary to develop AFLP markers, thus fingerprinting of any organism can be generated (Vos *et al.* 1995). In addition, AFLPs are highly polymorphic and highly reproducible (Jones *et al.* 1997). They can be detected and analyzed in a relatively short time and at low cost (Mueller and Wolfenbarger 1999). Due to all these characteristics, AFLPs are currently one of the most widely used molecular markers to study the genetic structure of natural populations (Bensch and Åkesson 2005, Meudt and Clarke 2007).

Previous studies employing AFLPs in amphibians have demonstrated that this is an effective technique to generate reliable markers for population level genetic inference (Curtis and Taylor 2003, Rogell *et al.* 2010, Zhang *et al.* 2011). Hence, in chapter 3 is presented the assessment and optimization of AFLP on the four frog species:

*Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1 and *Astylosternus* sp. 2 for which their genetic characterization is reported also in detail. Furthermore, the chapter 4 contains the genetic population structure of the four target species in Ngel Nyaki and Kurmin Danko Forest Reserve. This is of utmost importance because it is the first attempt to describe the genetic diversity and the genetic population structure of the

focal species, but also the first time the genetic effects of habitat fragmentation in continuous and fragmented populations have been investigated in detail for amphibians on the Mambilla Plateau or elsewhere in mountains of Cameroon and Nigeria. Finally, in the concluding chapter (chapter 5), the results from the three previous chapters are integrated. The importance of biological inventories particularly in biological hotspot is discussed as well as the relevance of the herpetofauna inventory on Ngel Nyaki. In addition, the criterion used to select the target frog species is discussed as are difficulties faced during the development of the project including polyploidy and interpretation of such results. Then, the utility of AFLP, reproducibility and the evaluation of genotyping error are also discussed. The results of genetic population structure for the four target species are examined to ultimately consider conservation priorities in terms of amphibian diversity as well as forest and riparian forest management on Mambilla Plateau. Finally, the necessity to explore the herpetofauna is discussed for nearby areas such as Gashaka Gumti National Park and other montane forests in Taraba State. In addition, the positive impact that this project had on the local community in Yelwa and Ngel Nyaki is discussed as is the necessity for continuing research -supported to some extent by locals- to extend the baseline data generated during this project.

#### **1.4 Species of interest**

Based on the herpetofaunal taxonomic survey conducted in and around the Ngel Nyaki and Kurmin Danko Forest Reserve (see chapter 2), three frog species of Arthroleptidae (*Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp.) were selected to

investigate the genetic effects of habitat fragmentation in amphibians on the Mambilla Plateau.

However, the compilation and preparation of data for reporting the preliminary list of the amphibians and reptiles of Ngel Nyaki led to confirmation that two species of *Astylosternus* occur here rather than one as first believed. Identification of the two species *A. rheophilus* and *A. diadematus* was supported with images and field data. Differences between adults are evident, but their tadpoles are similar morphologically making it difficult to differentiate them. Because most of the tissue samples collected for these species derive from tadpoles, species boundaries were delimited by using AFLP markers (see chapter 2) and a model-based clustering method based on the assumption that different species correspond to different genotypic clusters (Shaffer and Thomson 2007). The two species are hereafter referred to as *Astylosternus* sp. 1 and *Astylosternus* sp. 2. Species identification is still uncertain and further analysis using 12S and 16S mitochondrial DNA sequences will be conducted to assign species identity to the unknown individuals.

## **1.5 Criteria to select the target species**

This study is focused on Arthroleptidae that is the largest anuran family in the study area and from which the frog species *Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. were selected for carrying out an assessment of the population genetic structure. These species were chosen as they were frequently observed in the riparian forest fragments and continuous forest (see details below) enabling the comparison of different populations but also the collection of an adequate number of samples required for population genetics studies (Khanlou *et al.* 2011, Hale *et al.* 2012). Besides their



distribution and occupancy patterns within the study area, their conservation status was another significant factor. *Cardioglossa schioetzi* has been listed as Endangered, whereas *Leptodactylodon bicolor*, *Astylosternus diadematus*, and *A. rheophilus* are listed as Vulnerable on the IUCN Red List of Threatened Species (IUCN 2015). A common denominator for cataloguing these species on the IUCN Red List is their severely fragmented distribution, and the declining extent of their habitat in the montane forests of Cameroon and Nigeria.

*Cardioglossa schioetzi* was recorded mainly near streams in riparian forests outside the Ngel Nyaki Forest Reserve boundaries. A few specimens including tadpoles were caught at the edge of the forest near the field station of the Nigerian Montane Forest Project. Only a few males (not enough to complete an acceptable sample size for a population genetics study) were recorded calling in three different riparian forests on Kurmin Danko. *Leptodactylodon bicolor* as well as *Astylosternus* sp. 1 were sharing the same microhabitat, these two species were found together along the streams in the forest of Ngel Nyaki. *Astylosternus* sp. 1, was common and widely distributed within the forest and riparian forests on Ngel Nyaki and Kurmin Danko, whereas *A. sp. 2* was collected from forest in Ngel Nyaki. Interestingly, *L. bicolor* was found in only one riparian forest outside of the reserve, and no individuals were heard in Kurmin Danko (see figure 4-1 in chapter 4).

## **Chapter 2**

### **The amphibians and reptiles of Ngel Nyaki and Kurmin Danko Reserve, Mambilla Plateau, Nigeria**

#### **Abstract**

The herpetofauna of the montane forests of Taraba State in eastern Nigeria is poorly known. During the twentieth century a couple of surveys of amphibian and reptile diversity were conducted on the Mambilla Plateau, but neither compiled a comprehensive list of the amphibian and reptile species. Given patterns of diversity in the mountains of neighboring Cameroon, the mountains of Nigeria may also contain high species diversity, including both endemic and threatened species. A diversity of sampling methods such as active searching, Visual Encounter Survey (VES) and Acoustic Encounter Survey (AES) techniques, live pitfall traps, and opportunistic records were used to obtain the inventory. Here, I provide an annotated list of 21 amphibians and 11 reptiles collected on the Mambilla Plateau in or near both Ngel Nyaki and Kurmin Danko Forest Reserve. Nine of the 32 species listed here are new records for the Mambilla Plateau. While the lack of baseline data prevents the evaluation on the effect of the expanding anthropogenic impact and makes potential population declines difficult to determine, I document several likely threats to the amphibians and reptiles in this region. Ecological studies of habitat use and on-going monitoring of populations are needed as near-term conservation priorities.

## 2.1 Introduction

In many tropical regions the diversity of amphibians and reptiles is relatively well documented (Duellman 1999, Lips *et al.* 2005), however, in some parts of Central Africa it remains utterly unknown. Habitat loss and degradation are likely important drivers of population declines in this region (Stuart *et al.* 2004), and emerging disease may also prove a serious threat (Reeder *et al.* 2011, Doherty-Bone *et al.* 2013). The amphibian and reptile diversity of Cameroon has been better documented (Amiet 1972, Scott 1975, Amiet 1989, Herrmann *et al.* 2005, Chirio and LeBreton 2007, Blackburn 2008, Hirschfeld *et al.* 2012), whereas the fauna of neighboring Nigeria remains among the most poorly known in this region (Blackburn 2010).

The mountains of eastern Nigeria are part of the West African Forest biodiversity hotspot (Myers *et al.* 2000), and are regionally important in terms of biodiversity, including range-restricted and threatened endemic species. The mountains of the Obudu Plateau in Cross River State and the Mambilla Plateau in Taraba State are northwestern continuations of the Cameroon Volcanic Line, a geological structure comprising the mountains of Nigeria and Cameroon and the Gulf of Guinea Islands (Marzoli *et al.* 2000). The fauna and flora of these mountains is one of the most diverse on the continent (Burgess *et al.* 2004) and is found in a mosaic of montane forests and grasslands (>900 m elevation) that are part of the large Afromontane archipelago characterized by White (1983).

In Nigeria most of the montane forests are found in Taraba and Adamawa states in eastern Nigeria (Chapman and Chapman 2001). The Ngel Nyaki Forest Reserve includes Ngel Nyaki and Kurmin Danko forests, together comprising approximately 8km<sup>2</sup> of relatively undisturbed forest. They are satellite forests of the more extensive Godel Mountain forests in neighbouring Gashaka Gumti National Park (GGNP). Ngel Nyaki Forest

Reserve is floristically diverse and a refuge for several threatened plant species (Dowsett-Lemaire 1989, Chapman and Chapman 2001). It is also home to the Endangered Nigerian chimpanzee, *Pan troglodytes* subsp. *elliotti* (Beck and Chapman 2008), and several species of monkeys including the putty-nosed monkey (*Cercopithecus nictitans* cf. subsp. *martini*), mona monkey (*Ceropithecus mona*), and olive baboon (*Papio anubis*) (Chapman and Chapman 2001). In addition, it is a Birdlife International Important Bird Area (IBA) (Fishpool and Evans 2001 ).

Of all African habitats, montane forests are among the most susceptible to degradation and loss (Dunn 1999). As with many other habitats in Nigeria, the montane forests of Taraba State are threatened by expanding agricultural activities, human settlements, and overgrazing. The human population on the Mambilla Plateau has expanded over recent years. Hurault (1998) has suggested that between 1931 and 1981 the population increased from 16,201 to 126,184 inhabitants, and with that came an uncontrolled growth of livestock on the Mambilla Plateau. For instance, Leinde Fadali, a small forest within GGNP, has been negatively affected by an increase in settlements of Fulani cattle herders and their livestock. Pastures are located near the forest edge and cattle entering the forest cause soil compaction and erosion (Chapman *et al.* 2004, Bennett and Ross 2011). The situation is similar at Ngel Nyaki Forest Reserve, which is the focus of the herpetofaunal surveys presented here. Fulani pastoralists herd their cattle at the margins of the reserve, and occasionally within the boundaries, and perform annual burning of grasslands along the forest perimeter. Riparian forests outside of the reserves are also negatively impacted by these same activities. Streams are used as watering holes and cattle regularly cross these streams causing discontinuities in the riparian forests as well as compaction and erosion of the soil.

To date, taxonomic and distributional studies of Nigeria's herpetofauna are limited, especially in the mountains (Schiøtz 1963, Böhme and Nikolaus 1989, Blackburn 2010, Blackburn *et al.* 2010, Blackburn and Rödel 2011). This is true for Taraba's montane forests in GGNP including Chappal Hendu, Dutsin Lamba, Leinde Fadali, Chappal Waddi, Gangirwal (Chapman and Chapman 2001, Chapman *et al.* 2004), as well as Ngel Nyaki and Kurmin Danko forests (Blackburn 2010). Many species currently known only from Cameroonian mountains are likely found in Nigeria as well (see also Chirio and LeBreton 2007). More thorough surveys in the montane regions of Taraba State are needed as this region likely contains undescribed endemic biodiversity that may be threatened by habitat loss and degradation as described above (Blackburn 2010). Few surveys of amphibian and reptile diversity were conducted on the Mambilla Plateau during the twentieth century. Both Gartshore (1986) and Böhme and Nikolaus (1989) present a handful of herpetological records from on or near the Mambilla Plateau, but neither conducted large-scale surveys for amphibians and reptiles. Without baseline and longitudinal data, it is difficult to evaluate the long-term effect of the expanding anthropogenic effects on habitats in this region. Here I present an annotated list of the diversity of amphibians and reptiles in Ngel Nyaki Forest Reserve as well as comments on records from outside of the reserve such as *Eucalyptus* plantations and riparian forest fragments. These surveys provide northward range extensions of a number of montane species from Cameroon, including a number of threatened amphibian species.

## **2.2 Materials and Methods**

### **2.2.1 Study sites**

The surveys presented here were conducted over a two-week period in April 2009 and then more thoroughly from July to October during the rainy season of 2012. Collections and field surveys were conducted in and around the 46 km<sup>2</sup> Ngel Nyaki Forest Reserve (7°30'N, 11°30'E), including nearby Kurmin Danko forest (Figure 2-1). The forests occur at an elevation of 1400 to 1600 masl and contain ~7.5 km<sup>2</sup> of escarpment forest in which both hunting and tree felling have been illegal since 1969, the year in which it was gazetted as a Local Authority Forest Reserve (Chapman and Chapman 2001). In contrast, the nearby riparian forest fragments have no legal protection, and are therefore subject to extensive human disturbance. The mean annual rainfall of c. 1800 mm occurs mainly between mid-April and mid-October [Nigerian Montane Forest Project rainfall data]. Mean monthly maximum and minimum temperature for the wet and dry seasons are 26°C and 13°C, and 23°C and 16°C respectively [Nigerian Montane Forest Project (NMFP) records].

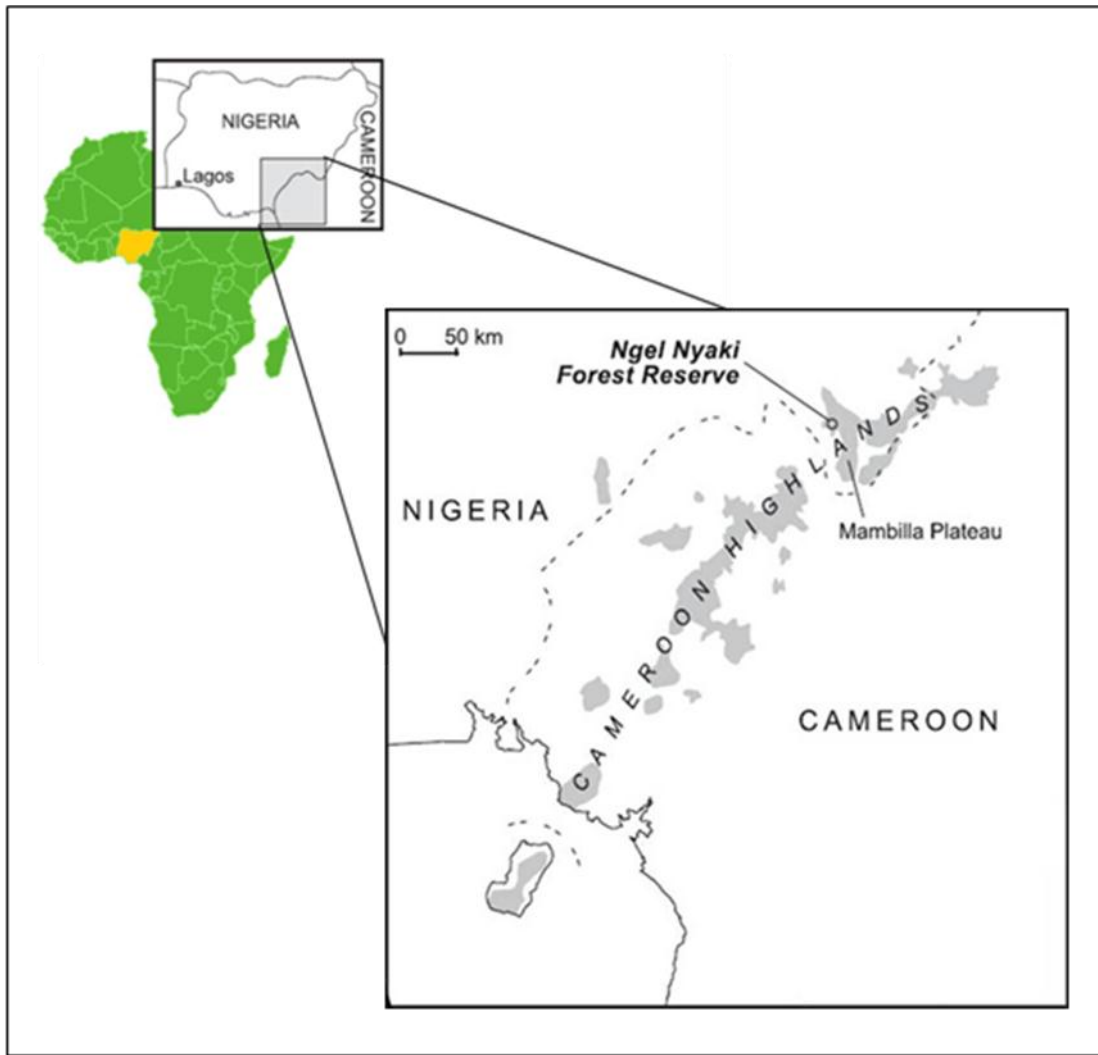


Figure 2-1. Location of Ngel Nyaki Forest Reserve on the Mambilla Plateau in eastern Nigeria (Adapted from Beck and Chapman, 2008).

Surveys reported here were conducted from the NMFP field station that sits within the boundaries of Ngel Nyaki Forest Reserve. The NMFP was created in 2004 to combine scientific research with education, involving not only national and international researchers, but also the local community to develop long-term sustainable management of the montane forests in Nigeria (NMFP 2012).

When Ngel Nyaki forest became a Forest Reserve, the village of Ngel Nyaki was located on the forest edge and within the reserve boundaries; the village was subsequently relocated and currently is known as Yelwa. This is the nearest village to the

reserve and its development, especially the expansion of outlying pastures and conversion of grasslands to farms, has negative effects on the reserve and associated riparian forests. Large areas around Yelwa have been planted with *Eucalyptus* spp. for human use (as firewood and building materials).

### **2.2.2 Sampling methods**

A diversity of sampling methods was used to obtain information for as many species as possible. The inventory was generated using active searching, Visual Encounter Survey (VES) and Acoustic Encounter Survey (AES) techniques, live pitfall traps combined with drift fences, and opportunistic records including roadkills and animals captured by people living nearby.

VES and AES are frequently used for rapid assessments and the evaluation of larger areas. These methods are better used as qualitative or semi-quantitative tools, therefore are useful for compiling an inventory on herpetofauna (Rödel and Ernst 2004). The VES technique consists of visually searching for animals in a given area for a prescribed amount of time (Crump and Scott 1994) providing information on the presence or absence of a species in an area (Corn and Bury 1990). As for the AES anuran males are detected through vocalization allowing for easy discrimination of species (Veith *et al.* 2004). Active searches and a combination of visual and acoustic techniques were conducted six days per week for 14 weeks in total. These techniques were carried out for at least four hours during the day and another four hours by the night in different habitat types: forest, riparian forest, grassland, *Eucalyptus* plantation, and disturbed areas (Figure 2-2).

Pitfall traps were used with drift fences as these arrays are more effective than traps on their own when sampling herpetofauna (Bury and Corn 1987). Pitfall traps consisted of



buckets sunk in the ground so that the upper rim was equal to or slightly below the ground-surface level. Small holes were made in the bottom of the traps to allow water to drain. Drift fences were made of plastic gauze tied/nailed to wooden stakes, 0.5 m high and at least 15 m long as recommended by Vogt and Hine (1982); traps or buckets were placed every 5m along the fence. These were buried up to 5 cm below the soil surface to prevent animals from burrowing under them. Trap systems were installed in different habitats including disturbed areas such as farms and areas where *Eucalyptus* has been cultivated. These traps were checked every day during both the early morning and evening.

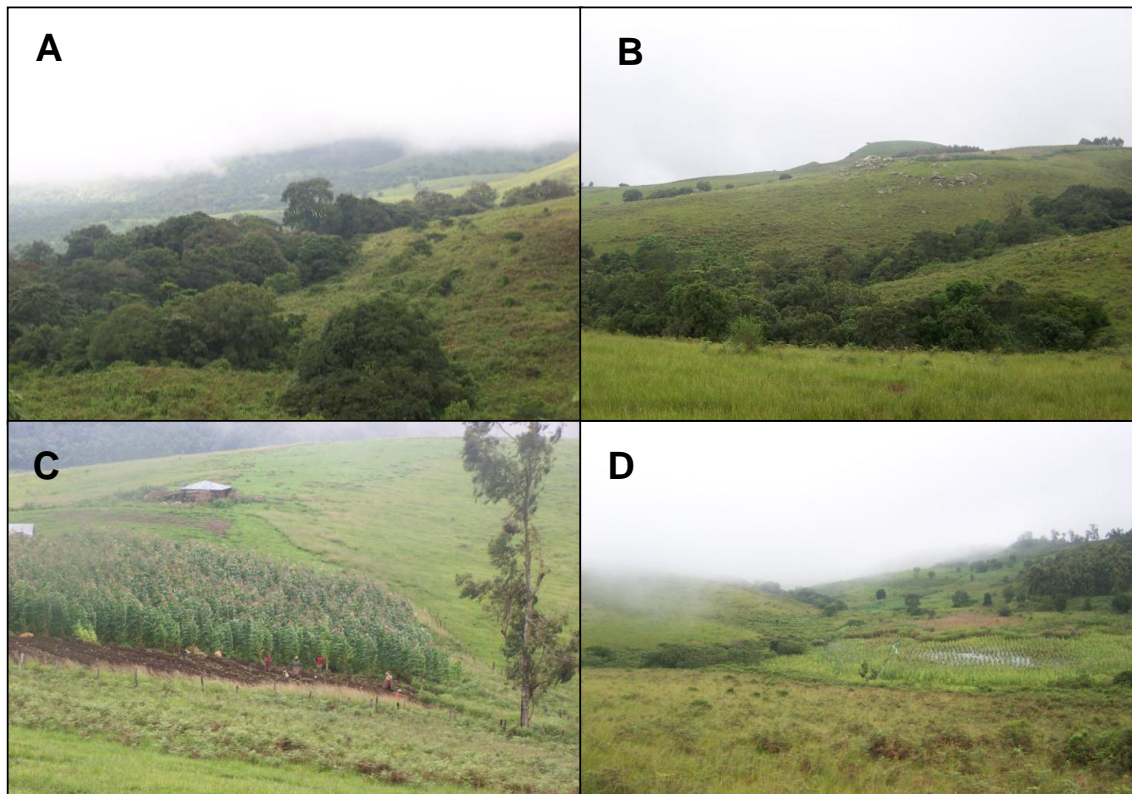


Figure 2-2. Examples of the types of habitats and disturbed areas. A) Edge of the forest reserve, B) Riparian forest, grassland, and rocky/bouldering areas, C) farm, and D) flooded corn fields.

Vouchering methods including photo documentation and collection of whole specimens were conducted to facilitate identifications and allow for comparisons to populations from other areas, especially in Cameroon. Amphibians were euthanized by immersion

in an aqueous solution of pH-neutralized Tricaine methanesulfanate (250 mg/liter, and buffered with sodium bicarbonate), also known as MS-222 (AVMA 2013). As for the reptiles a two-stage euthanasia method was employed. The first stage consisted of an intracoelomic injection (250-500 mg/kg) of pH-neutralized 1% MS-222, then, a second intracoelomic injection of unbuffered 50% MS-222 was applied (Conroy *et al.* 2009, AVMA 2013). Then, animals were preserved in 10% neutral-buffered formalin (Simmons 2002). Liver samples were taken from voucher specimens *post-mortem* and preserved in either 95% EtOH or RNALater.

Because the larvae of many African amphibians remain unknown, I also included sampling of tadpoles in this study. I used dipnets to capture tadpoles in a variety of habitats. Tissue samples of voucher larval specimens allow for identification of tadpoles using genetic data to match these from adults from the same or nearby localities.

Voucher specimens from 2009 were deposited at the Museum of Comparative Zoology (MCZ) at Harvard University, and those from 2012 will be deposited at the California Academy of Sciences (CAS; San Francisco, California, USA) (JVV field numbers), and at the Taraba State University (Jalingo, Nigeria) and the Nigerian Montane Forest Project field station (NMF field numbers) as research material and educational support. Data from 2012, specifically NMF field numbers were collected with approval of the Animal Ethics Committee of the University of Canterbury (UC) (permit Ref: 2012/24R). Although voucher specimens with field tags MCZ and JVV were not covered under this permit, they had a separate permit from the US and are cited here along with other data to complete the herpetofauna inventory as surveys were done in conjunction with other work groups as part of the same project.

Amphibian nomenclature generally follows Frost (2014) and Blackburn and Wake (2011). Reptile nomenclature generally follows that of Chirio and LeBreton (2007) with genus-level taxonomic updates as appropriate from the Reptile Database (Uetz 2010).

## **2.3 Results**

I recorded 21 amphibian and 11 reptile species. The amphibians represent six families and 12 genera of frogs. Of the reptiles, I recorded five snake species representing four families and six lizards representing five families.

Pitfall traps with drift fences were unsuccessful in most of the habitats; a total of 360 pitfall trap-days yielded just three captures corresponding to two species of anurans with two individuals trapped in the main forest near the field station and another in a *Eucalyptus* plantation near the village. Trap arrays installed outside the reserve in the grasslands and near the farms were seriously damaged by the cattle. After a few weeks, pitfall traps and drift fences were removed from all habitats and this method was abandoned.

I present below an annotated list of the amphibians and reptiles recorded in and around Ngel Nyaki and Kurmin Danko Reserve (Table 2-1). I use the terms rare and abundant to indicate relative rarity or abundance in the collection, though this may not reflect actual population sizes.

### **2.3.1 Species accounts**

#### **2.3.1.1 Amphibia**

##### **Anura**

##### **Arthroleptidae**

***Arthroleptis palava* Blackburn, Gvoždík & Leaché, 2010.** Voucher specimens: MCZ A-139527–28; JVV8009, 8112, 8126, 8164; NMF05. Specimens were recorded at the edge of the Ngel Nyaki forest, riparian forests, NMFP field station, grassland, *Eucalyptus* plantations, and in Yelwa Village.

This recently described species (Blackburn *et al.* 2010) is endemic to the mountains of the Cameroon Volcanic Line. The species was first collected, and even identified as probably a new species, in the late 1950s from the Obudu Plateau, but remained undescribed (see Blackburn *et al.* 2010). This species is abundant at the study site and may be the most common frog species. It is associated with anthropogenic landscapes; it is similarly found in disturbed habitats in Cameroon, for instance being found in farms of Elak-Oku on Mount Oku (DCB, pers. obs.).

***Arthroleptis cf. sylvaticus* Laurent, 1954.** Voucher specimens: MCZ A-139606; JVV8097, 8125. Specimens were collected at the edge of the Ngel Nyaki Forest Reserve. *Arthroleptis sylvaticus* is a widespread species associated with lowland and montane forest throughout much of Central Africa. Recent and on-going work indicates that populations typically referred to this species in fact represent a complex of several species, including the nominal species and one or several undescribed species (Blackburn 2008). The populations at Ngel Nyaki are likely conspecific with an undescribed species in the mountains of Cameroon (Figure 2-3A).

***Astylosternus cf. diadematus* Werner, 1898.** Voucher specimens: MCZ A-139562 (DCB 34676 and 34677; tadpoles), 139600, 139607, 139943, 139945; NMF03. Specimens were recorded in Ngel Nyaki and Kurmin Danko forests and their associated riparian forests. Individuals collected during the surveys are referable to either

*Astylosternus diadematus* or an undescribed species closely related to *A. diadematus* (Barej, Blackburn and colleagues, unpublished data). This same species was also found at lower elevations in Gashaka Gumti National Park (MCZ A-139568, 139628). Though populations of *A. diadematus* are abundant in suitable habitat, this species is considered Vulnerable because of its fragmented distribution and limited extent of occurrence (IUCN 2014).

***Astylosternus rheophilus* Amiet 1978.** Voucher specimens: MCZ A-139562 (DCB 34678; tadpole), 139564 (tadpole); JVV8022–23, 8100–103, 8181, 8183; NMF04, NMF11–12, 28, 31–32 (photo vouchers). An abundant frog species on the Ngel Nyaki Forest Reserve, during this study it was found in the main forest and riparian forest fragments near streams. The population at Ngel Nyaki is similar genetically to populations on Mount Oku, Tchabal Mbabo, and Mount Manengouba in Cameroon (Barej, Blackburn and colleagues, unpublished data). Although it is a common species, *A. rheophilus* populations present a declining trend (Stuart *et al.* 2008) perhaps as a consequence of the fragmented distribution of this species and the degradation of its habitat. These factors in addition to its small extent and area of occupancy lead this taxon to be considered Vulnerable. At Ngel Nyaki, this species co-exists in forested streams with *Leptodactylodon bicolor*.

***Cardioglossa pulchra* Schiøtz, 1963.** Voucher specimen: JVV 8065. A single male was captured on the grasslands near riparian forest, outside of Ngel Nyaki Forest Reserve. This species appears uncommon near Ngel Nyaki, although only one individual was found, calls were heard at other nearby sites adjacent to riparian forest fragments and where *C. schiøetzi* is often abundant. *Cardioglossa pulchra* is listed as Endangered

because it only occurs in less than 5,000 km<sup>2</sup> and its distribution has been severely fragmented with the increasing human population pressure within the mountains of Cameroon (IUCN 2014). In Nigeria, this species was previously recorded from the Cross River National Park and the Obudu Plateau (Schjøtz and Amiet, 2004). This is a new record for the Mambilla Plateau and significantly extends the northern range of this species (Figure 2-3B).

***Cardioglossa schioetzi* Amiet, 1981.** Voucher specimens: MCZ A-139561 (tadpole), 139946 (tadpole); JVV 8004–05, 8015–17, 8062–64, 8099, 8113–118, 8122; NMF18–19, photo voucher: NMF21–24, NMF33–36. While apparently widespread in riparian habitats in the mountains of Cameroon and Nigeria, this species remains poorly known with little information available on its ecology and population status (Stuart *et al.* 2008). Most of the specimens found in this study were near streams in riparian forest outside of Ngel Nyaki Forest Reserve boundaries but it was particularly abundant in riparian forest fragments close to the reserve. A few individuals including tadpoles were caught at the edge of the forest near the NMFP field station. Only a few males were recorded in the riparian forests at Kurmin Danko. Most of the specimens found during this survey are males (Figure 2-3C) that were found calling near streams at night (vocalization of specimen JVV8015 was recorded). A few males were heard calling during the day after a heavy rain. Calling activity was highest during dusk (17:30–18:30 H).

***Leptodactylodon bicolor* Amiet, 1971.** Voucher specimens: MCZ A-139563 (tadpoles), 139598–99, 139944 (tadpole); JVV8019, 8024–25, 8057–59, 8098, 8145–148, 8151; NMF01–02, 08–10, 13–17, 20, 27, 30 (Tadpoles). This species is common along the streams within Ngel Nyaki forest but not in the riparian forest fragments outside;

individuals were recorded in only one riparian forest outside the Reserve.

*Leptodactylodon bicolor* (Figure 2-3D) occurs in the same forested habitats as *Astylosternus* (see above). Male frogs vocalized both during the day and night. Because of deterioration of its forest habitats and limited extent of occurrence, *L. bicolor* is considered Vulnerable (Stuart *et al.* 2008).

***Leptopelis nordequatorialis* Perret, 1966.** Voucher specimens: MCZ A-139601, 139611–13; JVV8066–67, 8111, 8119–20, 8124; NMF37. This widespread species is found across the montane savannas of the Cameroon Volcanic Line, and is often associated with densely vegetated habitat near streams. Some of the specimens reported here were found buried at the NMFP field station in a plot with loose soil (used for organic waste disposal), which has also been documented in *Leptopelis viridis* (Schjøtz 1999). Other individuals were found on the trail near the NMFP field station at the edge of the Ngel Nyaki Forest Reserve, in riparian forest amidst large pastoral grasslands, and in farms near Yelwa (Figure 2-3E).

## **Bufonidae**

***Amietophrynus maculatus* (Hallowell 1854).** Voucher specimens: JVV8026, 8035–42, 8068–87. This is a widespread toad species in West and Central Africa that is frequently confused with *Amietophrynus regularis* (Poynton and Broadley 1988, Jackson and Blackburn 2007). It was found in the savanna but also at the NMFP field station and disturbed areas including *Eucalyptus* plantations and within Yelwa village. Many details are available on the biology of this species, including on its development, abundance, and behaviors (Rödel 2000).

***Amietophrynus* sp.** Voucher specimen: MCZ A-139614. This specimen was collected near the Ngel Nyaki forest. Genetic data reveals that this may be part of the larger *Amietophrynus gracilipes* complex, though it remains unclear whether this corresponds to a described species (DCB, unpublished data; E. B. Greenbaum, pers. Comm.)

***Amietophrynus villiersi* (Angel 1940).** Voucher specimens: MCZ A-139602–05, JVV8007–08, JVV8108. This species is found in montane savannas across the Cameroon Volcanic Line (Amiet 1972, Amiet 1989, Amiet and Tandy 2004). It was recorded in the NMFP field station, near farms, *Eucalyptus* plantations and tracks nearby the village. All the specimens were collected in or near Yelwa village.

## **Hyperoliidae**

***Afrixalus quadrivittatus* (Werner, 1908).** Voucher specimens: JVV 8091–92. Individuals were found in open flooded grasses and corn fields near the village. Following Perret (1976) and Rödel (2000), *Afrixalus* found at Ngel Nyaki was recognized as *A. quadrivittatus*. Amiet (2012) refers to this same species as *A. fulvovittatus brevipalmatus*, and additional work is needed to investigate species boundaries between West and Central African populations.

***Hyperolius igbettensis* Schiøtz, 1963.** Voucher specimens: JVV 8127–32, 8168–69. It is relatively common in the flooded corn fields near Yelwa. This widespread species occurs from western Africa to Cameroon and possibly further east (Amiet 2005, Amiet 2012, Channing *et al.* 2013). In neighboring Cameroon this species has a wide elevational range (~400–2000 masl) and while its distribution is incompletely known it seems largely associated with habitats dominated by grasses (Amiet 2012).



***Hyperolius nitidulus* Peters, 1875.** Voucher specimens: JVV8133–39, NMF38 (photo voucher). Specimens were collected near Yelwa in a flooded farm next to a stream in grassland and another individual (NMF38) was found outside the Ngel Nyaki Forest Reserve within the savanna. This species is widespread in the highlands of neighboring Cameroon (Amiet 2012). Amiet (2012) considers this taxon as a distinct species, whereas Schiøtz (1971) recognized it as *Hyperolius viridiflavus aureus* and thus part of the larger *Hyperolius viridiflavus* complex (Figure 2-3F).

***Hyperolius riggenbachi* Nieden, 1910.** Voucher specimens: NMF06–07, 25–26. Individuals were recorded outside of the forest in riparian forest fragments in both Ngel Nyaki and Kurmin Danko. Specimens were also collected in riparian forests nearby areas opened through forest fragments by the transit of cattle. This species is widespread throughout the montane savannas of the Cameroon Volcanic Line and is well-known for its striking sexual dichromatism.

***Kassina decorata* (Angel, 1940).** Voucher specimens: JVV8088–89, 8123. Specimens were collected near Yelwa on grasses along the road. Specimens were collected close to the main road just north of Yelwa. This species was identified based on Amiet's (2012) diagnosis, including the fusion of the supratympanic and post-tympanic spots (sensu Amiet 2012). This species has previously only been reported from the savanna in the Mount Manengouba caldera and on the Adamoua Plateau (Figure 2-3G) in neighboring Cameroon.

## **Phrynobatrachidae**

***Phrynobatrachus danko* Blackburn, 2010.** Voucher specimens: MCZ A-139529–33.

A recently described frog species that is the only amphibian species so far known only from the Mambilla Plateau (Blackburn 2010). This puddle frog was collected in the forest of Kurmin Danko during 2009; specimens of this species have not been found either in Ngel Nyaki or in riparian forests close to Kurmin Danko. To date, only one female has been collected (MCZ A-139530). In 2012 this species was not seen, however, data from 2009 is used to complete the herpetofauna inventory.

***Phrynobatrachus steindachneri* Nieden, 1910.** Voucher specimens: MCZ A-139608–09, 139565 (tadpole). Individuals were collected at the Ngel Nyaki Forest Reserve. This puddle frog species has been recently restricted to populations from the mountains of northern Cameroon and the Mambilla Plateau and Gotel Mountains of eastern Nigeria (Zimkus and Gvoždík 2013). The specimens collected here are most similar genetically to populations from the Gotel Mountains (Zimkus and Gvoždík 2013).

## **Pipidae**

***Xenopus* sp. (*fraseri*-subgroup).** Voucher specimens: JVV8050–52; NMF29. Tadpoles were collected from a pool at the edge of the Ngel Nyaki Forest Reserve (where water is collected for the field station). A few adults were found also in Kurmin Danko along streams in riparian forests. Identification of species in the *fraseri*-subgroup is challenging because of the morphological similarity among most species and identification will require comparing genetic data to populations from nearby Cameroon where this subgroup is particularly diverse.

***Xenopus laevis* (Daudin, 1802).** Voucher specimens: JVV8090, 8093–95. A widespread and well-known clawed frog species (Figure 3H). In Ngel Nyaki, this species was found in a pool, whereas in Yelwa it was recorded on flooded grasses near the main road. It is a model organism in developmental and molecular biology (Cannatella and de Sá 1993) and there are feral populations in Europe and the Americas.

### **Ptychadenidae**

***Ptychadena* sp.** Voucher specimen: MCZ A-139610. The taxonomic status of the specimen found in Yelwa remains uncertain. Delimitation among species of *Ptychadena* is not straightforward and the taxonomy is currently undergoing revision (e.g. Dehling and Sinsch 2013).



Figure 2-3. Examples of the frog species collected in and around Ngel Nyaki forest. A) *Arthroleptis* cf. *sylvaticus* (JVV8097), B) *Cardioglossa pulchra* (JVV8065), C) *Cardioglossa schioetzi* (JVV8004), D) *Leptodactylodon boicolor* (JVV8019), E) *Leptopelis nordequatorialis* (JVV8067) F) *Hyperolius nitidulus* (JVV8133), G) *Kassina decorata* (JVV8089), and H) *Xenopus laevis* (JVV8090). (Images by D.C. Blackburn).

Table 2-1. Localities where species were recorded (Ngel Nyaki, including the forest, associated riparian forests and grassland; same for Kurmin Danko. Yelwa includes the village, corn fields, and flooded areas). Conservation status following the IUCN Red List category. Least Concern (LC), Vulnerable (VU), Endangered (EN), Data Deficient (DD). New records for Nigeria (N) and Mambilla Plateau (M).

Species	Localities			IUCN Red List category	New records
	Ngel Nyaki	Kurmin Danko	Yelwa		
<b>Amphibia</b>					
<b>Arthroleptidae</b>					
<i>Arthroleptis palava</i>	x		x	LC	
<i>Arthroleptis</i> cf. <i>sylvaticus</i>	x			LC	M?
<i>Astylosternus</i> cf. <i>diadematus</i>	x	x		VU	N, M
<i>Astylosternus rheophilus</i>	x	x		VU	N, M?
<i>Cardioglossa pulchra</i>	x			EN	M
<i>Cardioglossa schioetzi</i>	x	x		EN	M
<i>Leptodactylodon bicolor</i>	x			VU	N?, M
<i>Letopelis nordequatorialis</i>	x	x	x	LC	
<b>Bufonidae</b>					
<i>Amietophrynus maculatus</i>	x		x	LC	
<i>Amietophrynus</i> sp.	x				
<i>Amietophrynus villiersi</i>	x		x	EN	
<b>Hyperoliidae</b>					
<i>Afrixalus quadrivittatus</i>			x	LC	M
<i>Hyperolius igbettensis</i>			x	LC	N, M?
<i>Hyperolius nitidulus</i>	x		x	LC	
<i>Hyperolius riggenbachi</i>	x	x		VU	
<i>Kassina decorata</i>			x	DD	N, M
<b>Phrynobatrachidae</b>					
<i>Phrynobatrachus danko</i>		x		-	
<i>Phrynobatrachus steindachneri</i>	x			VU	M

Table 2-1 continued from previous page

Species	Localities			IUCN Red List category	New records
	Ngel Nyaki	Kurmin Danko	Yelwa		
Pipidae					
<i>Xenopus</i> sp. ( <i>fraseri</i> -subgroup)	x	x			
<i>Xenopus laevis</i>	x	x		LC	M
Ptychadenidae					
<i>Ptychadena</i> sp.			x		
Reptilia					
Colubridae					
<i>Philothamnus angolensis</i>	x				
Lamprophiidae					
<i>Lycophidion multimaculatum</i>	x				
Typhlopidae					
<i>Rhinotyphlops decorosus</i>	x		x		M?
Viperidae					
<i>Atheris squamigera</i>	x				
<i>Bitis gabonica</i>	x				
Agamidae					
<i>Agama lebretoni</i>	x				N, M?
Chamaeleonidae					
<i>Trioceros wiedersheimi</i>	x				
Gekkonidae					
<i>Cnemaspis spinicollis</i>	x				M?
<i>Hemidactylus albituberculatus</i>			x		
Scincidae					
<i>Trachylepis</i> “sp. 4”	x				M?
Varanidae					
<i>Varanus niloticus</i>	x				

### 2.3.1.2 Reptilia

#### Colubridae

***Philothamnus angolensis* Bocage, 1882.** Photo voucher: NMF39. This is a common species of Central and southern Africa. It is a tree-dwelling species that feeds on small lizards and amphibians (Chirio and LeBreton 2007). A specimen was recorded at the NMFP field station; it was seen attacking a chick of weaverbird (*Ploceus cucullatus*) in an avocado tree.

#### Lamprophiidae

***Lycophidion multimaculatum* Boettger, 1888.** Voucher specimen: JVV8167. This widespread savanna species occurs across a wide elevational range in Central, southern, and eastern Africa (Chirio and LeBreton 2007). This specimen was found at the NMFP field station (Figure 2-4A).

#### Typhlopidae

***Rhinotyphlops decorosus* Buchholz and Peters, 1875.** Voucher specimens: JVV 8096, 8158, 8162. This species occurs in degraded forest and forest-savanna mosaic across a broad elevational range in neighboring Cameroon (Chirio and LeBreton 2007). Three specimens were collected, two of which were found in Yelwa village and the third within the NMFP field station, suggesting that while potentially uncommon they are tolerant of disturbance around Ngel Nyaki (Figure 2-4B).

## **Viperidae**

*Atheris squamigera* (Hallowell, 1854). Voucher specimens: JVV 8060–61. Specimens were collected at night resting on branches of trees within the forest of Ngel Nyaki near a stream. This widespread forest species (Figure 2-4C) is found throughout West and Central Africa (Chirio and LeBreton 2007). This species faces conservation problems due to deforestation of forested areas (Chirio and LeBreton 2007).

*Bitis gabonica* (Duméril, Bibron and Duméril, 1854) Photo voucher: NMF40. An individual was recorded in the forest of Ngel Nyaki.

## **Agamidae**

*Agama lebretoni* Wagner, Barej, and Schmitz 2009. Voucher specimens: JVV8006, 8027–28, 8043–49, JVV8109–110, 8152–156, JVV8159–161, JVV8171–180. Most of the specimens were found outside of Ngel Nyaki Forest Reserve boundaries on boulders in open grasslands near the NMFP field station (Figure 2-4D). These are the first confirmed records of *A. lebretoni* in Nigeria though it is expected to occur more widely. This recently described species is common across Cameroon and extends into Gabon (Wagner *et al.* 2009) and was previously discussed as *Agama* “sp. 1” by Chirio and LeBreton (2007).

## **Chamaeleonidae**

*Trioceros wiedersheimi* (Nieden, 1910). Voucher specimens: JVV8010–14, 8054–56, 8107, 8121, 8140–143, 8150. A vulnerable species that is restricted to the highlands of western Cameroon and eastern Nigeria (Akani *et al.* 2001, Gonwouo *et al.* 2006) and frequently exported for the pet trade (Chirio and LeBreton 2007) (Figure 2-4E).



Specimens were found on the forest, on the trail near the NMFP field station at edge of the forest, and grassland outside of the reserve.

### **Gekkonidae**

***Cnemaspis spinicollis* (Müller, 1907).** Voucher specimen: JVV8104. This forest-specialist species is identifiable by the prominent white conical spine on the neck and is found at a wide elevational range in neighboring Cameroon (Chirio and LeBreton 2007). An individual was captured on a large boulder over a stream within Ngel Nyaki forest near the NMFP field station (Figure 2-4F).

***Hemidactylus albituberculatus* Trape, 2012.** Voucher specimen: JVV8144. This recently described species is found in savanna (and frequently on houses) from Togo to western Cameroon (Trape *et al.* 2012). A specimen was brought to the NMFP field station by a local from Yelwa, but surprisingly none were recorded on buildings at the field station (Figure 2-4G).

### **Scincidae**

***Trachylepis* “sp. 4” sensu Chirio & LeBreton, 2007.** Voucher specimens: JVV8018, 8020–21, 8029–30, 8105–106, 8157, 8170. Populations at Ngel Nyaki appear to be conspecific with those reported recently as *Trachylepis* “sp. 4” by Chirio and LeBreton (2007). This undescribed species has been previously confused with *T. perroteti* and is widespread in savannas of northern Cameroon (Chirio and LeBreton 2007). It is a common species around the NMFP field station and most specimens were captured near the field station. One individual was collected in a rock outcrop in open grassland near the Ngel Nyaki Forest Reserve (Figure 2-4H).

## **Varanidae**

***Varanus niloticus* (Linnaeus, 1766).** Photo vouchers: NMF43–44. Nile monitors are common across much of Africa and are well known from neighboring Cameroon (Chirio and LeBreton 2007). Two individuals were observed in Ngel Nyaki Forest Reserve. Though some non-Muslim ethnic groups eat this species, populations of this species are not believed to be threatened (Chirio and LeBreton 2007).

### **2.3.2 Conservation status**

Nine (47%) out of the 21 recorded amphibians species are listed as Least Concern in the IUCN Red List (IUCN 2015) , and five others (26%) are classified as Vulnerable. Only three species (16%) (*Cardioglossa pulchra*, *Cardioglossa schioetzi* and *Amietophrynus villiersi*) are considered Endangered (Table 2-1) because their distribution has been fragmented, their habitat in Nigeria and Cameroon has been declining, and their extent of occurrence is probably less than 5,000 km<sup>2</sup> (IUCN 2015). For the other species recorded, two are still of uncertain taxonomic status, one has not yet been assessed for the IUCN Red List, and another is considered Data Deficient.

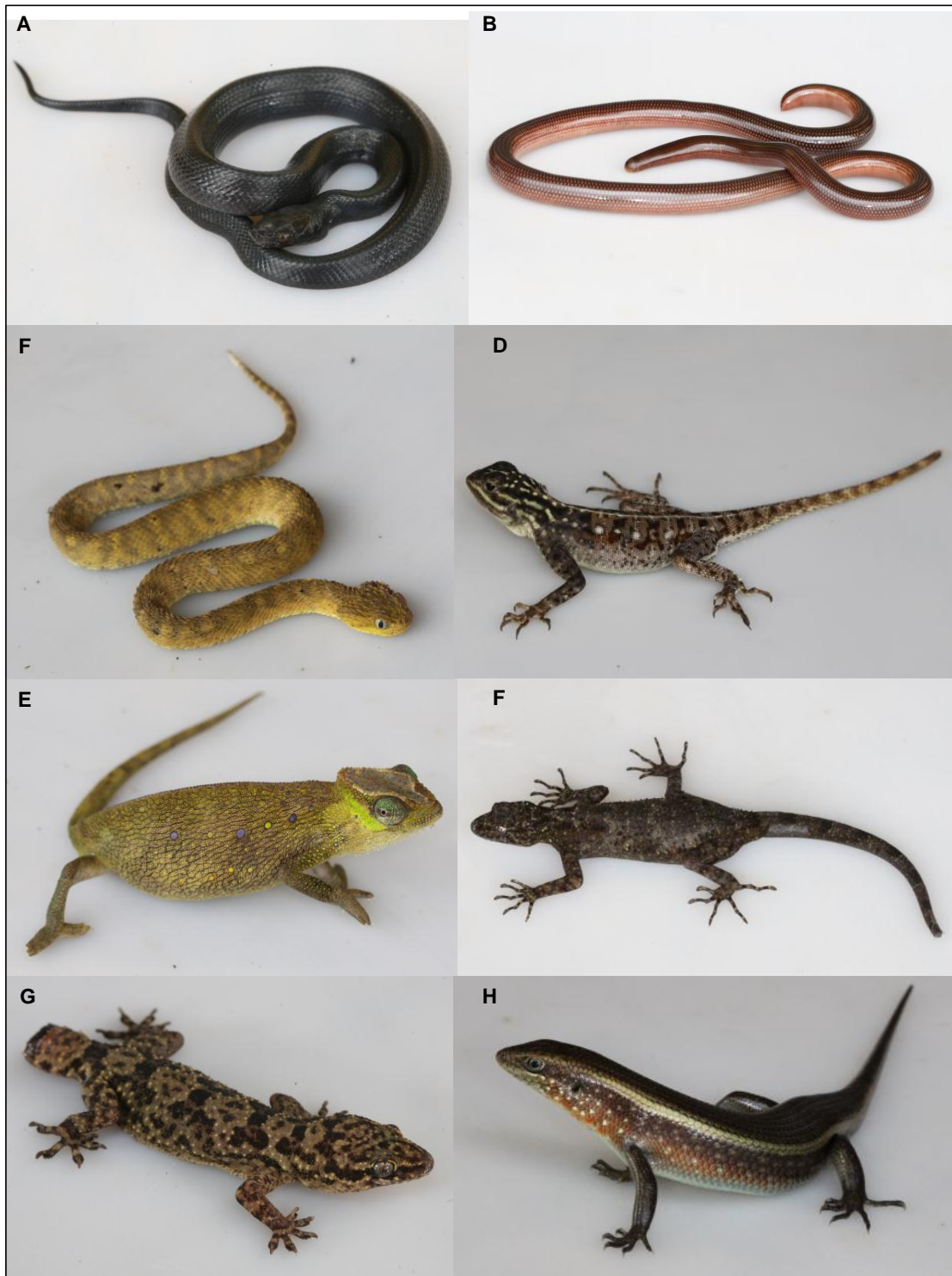


Figure 2-4. A) *Lycophidion multimaculatum* (JVV8167), B) *Rhinotyphlops decorosus* (JVV8096), C) *Atheris squamigera* (JVV8061), D) *Agama lebretoni* (JVV8006), E) *Trioceros wiedersheimi* (JVV8054), F) *Cnemaspis spinicollis* (JVV810), G) *Hemidactylus albituberculatus* (JVV8144), and H) *Trachylepis* “sp. 4” (JVV8105). (Images by D.C. Blackburn).

None of the 11 reptile species recorded during this survey have been listed in the IUCN Red List, except for *Trioceros wiedersheimi* which has been classified as Data Deficient. As a species of *Trioceros*, this species is included within CITES Appendix II.

## 2.4 Discussion

While undoubtedly incomplete, these surveys represent the most thorough inventory to date of amphibians and reptiles on the Mambilla Plateau of eastern Nigeria. Previous surveys conducted in 1989 as part of a multidisciplinary expedition to eastern Nigeria recorded seven anuran species, but only two of these (*Amietophrynus maculatus* and *Leptopelis nordequatorialis*) were recorded from Ngel Nyaki Forest Reserve (Böhme and Nikolaus 1989). Twenty years later, in 2009, a rapid survey on Ngel Nyaki and Kurmin Danko forests produced a number of the records included here as well as the description of two new frog species: *Arthroleptis palava* (Blackburn *et al.* 2010) and *Phrynobatrachus danko* (Blackburn 2010).

Apart from those relatively widespread species occurring in the savanna and disturbed areas, such as the anurans of the genus *Amietophrynus*, or the snake species *Lycophidion multimaculatum*, the records from Ngel Nyaki and Kurmin Danko include a few remarkable findings. I recorded the frogs *Astylosternus* cf. *diadematus*, *A. rheophilus*, *Leptodactylodon bicolor*, *Hyperolius igbettensis*, and *Kassina decorata* for the first time in Nigeria. Likewise, I documented here the first confirmed records of *Agama lebretoni* in Nigeria though it is expected to occur more widely. Six other anuran species (*Arthroleptis* cf. *sylvaticus*, *Cardioglossa pulchra*, *C. schioetzi*, *Afrixalus quadrivittatus*, *Phrynobatrachus steindachneri*, and *Xenopus laevis*) and three reptile species (*Rhinotyphlops decorosus*, *Cnemaspis spinicollis*, *Trachylepis* “sp. 4”) are reported for the first time in the Mambilla Plateau. Some of these species are

particularly of note since their occurrence in Ngel Nyaki and Kurmin Danko significantly increases the current knowledge of their distribution. This includes the threatened frog species *Cardioglossa pulchra* the range of which is now known to extend into Taraba State of Nigeria.

Notably, species of other genera such as *Werneria* and *Conraua* that occur at mid- and high elevations in Cameroon were not observed in the study area. It is important to note that several species of these unrecorded genera are from primary and slightly disturbed forest, requiring a closed canopy and thus are likely intolerant of habitat degradation (Stuart *et al.* 2008). Further studies are needed to determine whether species of these genera are present here or elsewhere on the Mambilla Plateau.

#### **2.4.1 Conservation and management considerations**

The montane forests in Taraba State are threatened by anthropogenic activities including burning and land clearance for farming (Chapman and Chapman 2001). Even though Ngel Nyaki and Kurmin Danko have been gazzeted as reserve, the nearby riparian forests are not included and have therefore been subject to more extensive human disturbance.

By conserving forest stream habitats, the persistence of several species including the anurans *Astylosternus rheophilus*, *A. cf. diadematus*, *Leptodactylodon bicolor*, and the forest-restricted lizard *Cnemaspis spinicollis* will be ensured. Although none of the frog species reported here is restricted to forest, it is worth noting that *Leptodactylodon bicolor*, which was common along the streams within Ngel Nyaki Forest Reserve, was recorded from only one riparian forest fragments outside the reserve. Efforts should be considered for protecting and possibly reforesting smaller riparian forest fragments that are key sites for some anuran species such as *Cardioglossa schioetzi*.

The open savanna/grassland montane habitat is essential for some of the species listed here, for instance, the amphibians *Amietophrynus villiersi*, *Leptopelis nordequatorialis* and *Hyperolius nitidulus* and for the reptiles *Agama lebretoni*, *Trioceros wiedersheimi*, and *Trachylepis* “sp. 4”. Some of these species do not occur in montane forests and are probably restricted to montane grasslands. This is true for the frog *Kassina decorata* which was previously known only from savannas in the Mount Manengouba caldera and on the Adamawa Plateau in Cameroon. On the Mambilla Plateau these habitats are under threat from cattle and burning. Thus, the herpetofauna inhabiting predominantly or exclusively these grasslands should be considered at risk. In light of threats to both grassland and forest species, immediate conservation actions are needed to monitor their populations. The present study provides insight into the herpetofauna from the montane forest in the Mambilla Plateau, but other montane forests in Taraba State remain unexplored. This includes Chappal Waddi and Chappal Hendu within the Gashaka Gumti National Park for which field surveys are needed as hardly anything is known of the amphibian and reptile fauna in this region.

Because of the lack of previous base-line data on the herpetofauna at Ngel Nyaki, it is difficult to evaluate population trends, including population declines, or to make comparisons to the amphibian and reptile composition in other nearby areas.

Unfortunately, population declines of several amphibian and reptile species are highly probable in this area as Ngel Nyaki Forest Reserve is threatened by burning and cattle encroachment (Chapman *et al.* 2004), and the riparian forest fragments and grassland are already extremely impacted. Beyond the boundaries of the reserve there is no legal protection, and the grasslands are constantly crossed by cattle that cause compaction and erosion of the soil. In the riparian areas, the cattle also cause discontinuities between riparian forests. In addition, human activities exacerbate this degradation

through contamination of the streams which results in poor water quality. Kurmin Danko is in a similar but perhaps more severe situation as this area has only recently been gazzeted.

To address conservation priorities, it is essential to produce information on the abundance, life history, distribution, and genetics of species. In addition, it is necessary to keep continuous monitoring programs to detect and mitigate population declines but also possible subsequent extinctions of the herpetofauna in this area. Research on the ecology and composition of the amphibian and reptile species will continue at Ngel Nyaki and Kurmin Danko forests to gradually add information to what is already known. Moreover, herpetofauna surveys will be extended to other montane forests in the Taraba State. New findings will increase substantially the knowledge on composition and distribution of the Central African herpetofauna and contribute towards effective conservation decisions.

## Chapter 3

### **An assessment and optimization of the Amplified Fragment Length Polymorphism (AFLP) method for the genetic characterization of four African anuran species**

#### **Abstract**

Little is known about the amphibian species inhabiting the montane forest on the Mambilla Plateau, Nigeria, where anthropogenic activities such as farming and cattle raising are a major threat to native biodiversity. It is urgent to establish conservation priorities for this group, yet essential information necessary for this is missing; no data on the genetic structure of any amphibian species in this part of Africa exists. In this chapter, the use of Amplified Fragment Length Polymorphisms (AFLP) to genetically characterize four poorly known frog species (*Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1 and *Astylosternus* sp. 2) inhabiting the Ngel Nyaki Forest Reserve and surrounds was assessed. Two AFLP primer combinations yielded 631 fragments out of which 554 were polymorphic. The maximum number of scored polymorphic peaks was 108, recorded for *A. sp1*. The average genotyping error rate per locus was 6.38% for the two preferred primer pairs. The Polymorphic Information Content or ‘informativeness’ of the two markers ranged between 0.21 and 0.28 and demonstrated their use for future population genetic studies. Results of this study show



that AFLP is a valuable tool when no prior molecular/genetic information of a species is available. Moreover, these two markers have proved to be informative enough to be used for further analysis at a population genetic level for the four target species.

### **3.1 Introduction**

The amphibian diversity of Nigeria's montane forests remains poorly understood (Blackburn 2010) and consequently, the genetic composition and structure of populations of a number of species remain unexplored. This is especially worrying as amphibians on the Mambilla Plateau are threatened by expanding agricultural activities, human settlements, and overgrazing. Under these intense time constraints it is necessary to rapidly establish conservation priorities. For these to be most useful genetic information is imperative as it results in better informed decisions (Storfer 2003, Storfer *et al.* 2009).

One of the major goals of conservation biology is to identify and protect the evolutionary heritage and future evolutionary potential in threatened species (Monsen and Blouin 2003). Molecular markers are a valuable tool in identifying populations of a particular species that deserve priority in conservation (Petit *et al.* 1998). The use of molecular markers has produced genetic data that has allowed the assessment of the population structure, the impact of genetic drift on genetic variation, the level of inbreeding within populations, and the amount of gene flow between and within populations (Ouborg *et al.* 2010). Thus, genetic data can guide management strategies to preserve genetic diversity and to promote and maintain genetic connectivity of populations of endangered species (Richards-Zawacki 2009).

A number of molecular markers are available to assess population genetic structure, including Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and microsatellites or Simple Sequence Repeats (SSR) (Sunnucks 2000, Arif and Khan 2009). For some African amphibian species there is already some genetic information as DNA sequences (e.g. mitochondrial DNA sequence data from 12S rRNA, and 16S rRNA) have been produced from several systematic and biogeographic studies on West African amphibians (e.g. Blackburn 2008, Zimkus 2009). However, specific microsatellite markers have been isolated only for one African anuran species (within the family Phrynobatrachidae) (Sandberger *et al.* 2010), and an African caecilian species (Gymnophiona) (Barratt *et al.* 2012). Microsatellites are highly polymorphic and abundant, easily amplified by Polymerase Chain Reaction (PCR), thus, a versatile tool for molecular fingerprinting (Arif and Khan 2009). Although microsatellites are highly informative, and cross-species amplification is possible between closely related species (Nair *et al.* 2012), their high mutation rates prevent the design of primers for universal application and therefore species-specific microsatellite markers need to be isolated (Arif *et al.* 2011). The development of new specific primers is costly and time consuming (Abdelkrim *et al.* 2009), rendering microsatellites as an inaccessible tool when time and financial resources for a given study are limited (Jehle and Arntzen 2002). No species-specific microsatellites have been isolated yet for any of the species within the anuran family Arthroleptidae of which the four target species in this study are members.

AFLP (see below) is currently one of the most widely used molecular markers to study the genetic structure of natural populations (Bensch and Åkesson 2005, Meudt and Clarke 2007). The technique is based on the detection of genomic restriction fragments

by PCR amplification using arbitrarily selected primers, so that it is able to generate fingerprints in any DNA sample regardless of its genome size. AFLP markers have several advantages over other molecular markers. For instance, no prior DNA sequence information is necessary to develop AFLP markers, thus fingerprinting of any organism can be generated (Vos *et al.* 1995); in addition, they contain a high level of polymorphisms, are highly reproducible (Jones *et al.* 1997) and can be detected and analyzed in a relatively short time and at low cost (Mueller and Wolfenbarger 1999). Historically AFLP has been used mostly for plants, fungi and bacteria (Bensch and Åkesson 2005), but more recently has been applied to animal genomes (e.g. Kazachkova *et al.* 2004, Liu *et al.* 2009, Han *et al.* 2013). Previous use of AFLP in amphibians suggests that this is an effective technique to generate reliable markers for population level genetic inference (Curtis and Taylor 2003, Rogell *et al.* 2010, Zhang *et al.* 2011).

The aim of the present study was to assess the utility of AFLP to genetically characterize four frog species: *Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1 and *Astylosternus* sp. 2. More information on the ecology and distribution of these species can be found in chapters 2 and 4. Due to the lack of genetic information of the target species it was critical in the first place to obtain base-line data to be used for further studies on population genetics. In this case the data generated from this study was later used to inform a study focused on measuring the genetic population structure of these frog species and assessing the impact of habitat fragmentation on connectivity among their populations (see chapter 4).

### **3.1.1 Principle of the AFLP technique**

The AFLP method is based on the selective amplification of fragments of total digested genomic DNA (Figure 3-1). The original protocol was designed to detect fragments on denaturing polyacrylamide gels, however, nowadays the most used detection systems is through automated sequencer devices by using fluorescently labeled primers.

The first stage of the AFLP method consists of template preparation by digesting the DNA. The aim of this first step is to produce a mix of many fragments from total genomic DNA. To do this, two different restriction enzymes are used, a frequent cutter that produces small fragments, and a rare cutter, which reduces the number of fragments to be amplified. Once the digestion is completed three types of fragments are produced. Fragments with rare cuts at both ends (commonly the longest fragments), fragments with frequent cuts at both ends (smallest fragments), and fragments with a rare cut at one end and a frequent cut at the other end. These particular fragments are predominately amplified, as the technique was developed to preferentially detect them (Vos *et al.* 1995).

Digestion is followed by a ligation step in which T4 ligase enzyme is used to join double-stranded linkers to the restriction fragments. AFLP linkers or adapters are oligonucleotides that are in part complementary to each other and form a double-stranded structure. They are designed in such a way that ligation of a fragment does not reconstitute the restriction site. The structure of AFLP adapters comprises a core sequence and a specific sequence for the enzyme. Due to the particular design of such linkers, digestion and ligation might be performed in the same reaction as long as the restriction and the T4 ligase enzymes are active at the same temperature (Bonin *et al* 2005).

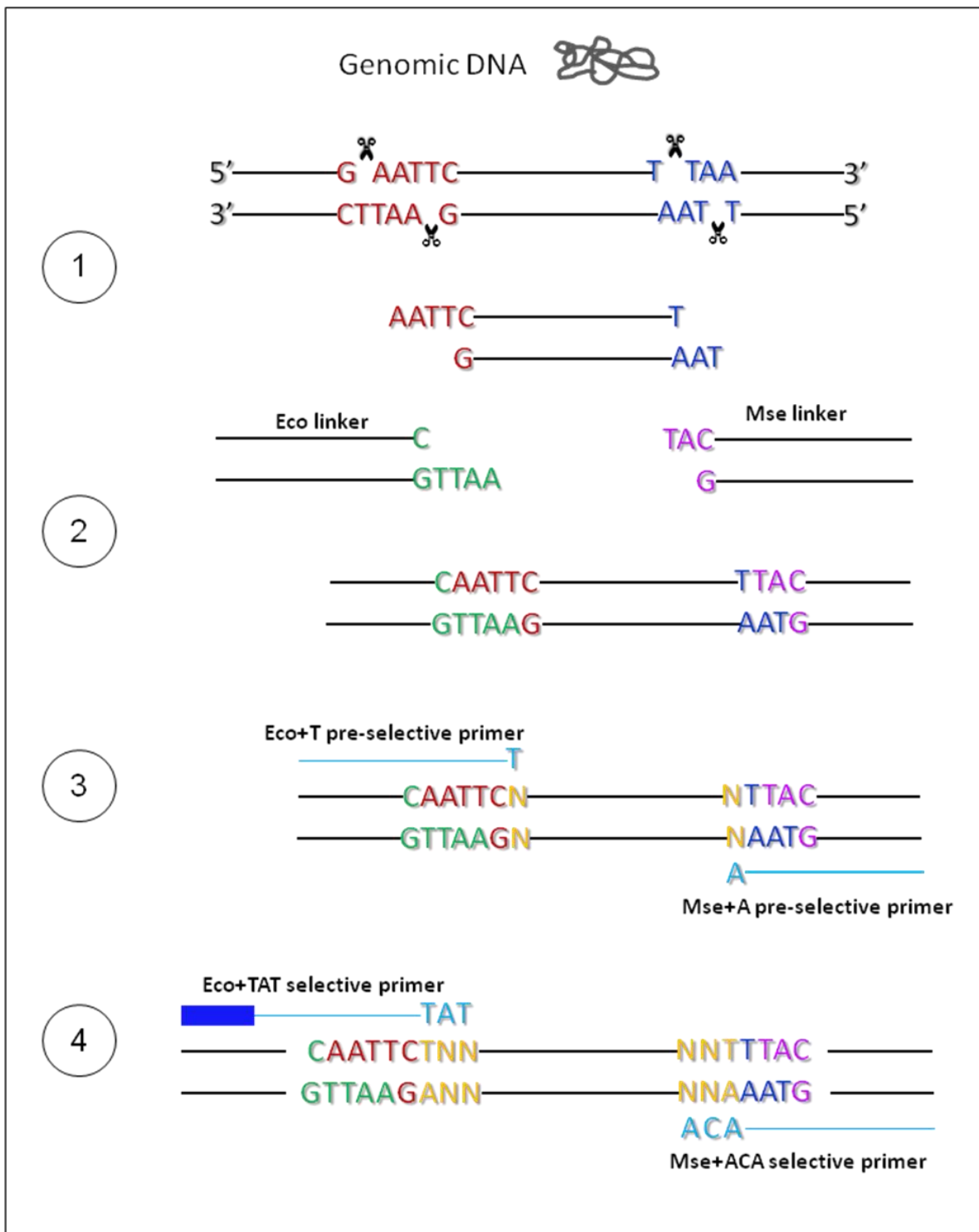


Figure 3-1. The AFLP technique is based on selective amplification of a subset of DNA fragments produced by restriction enzymes. 1) Double digestion of the genomic DNA. 2) Ligation of adapters and restriction fragments. 3) First amplification or Pre-selective amplification. 4) Selective amplification. After these steps, AFLP fragments are detected by means of capillary separation using automated DNA sequencers.

In the third step the complexity of the ligated fragments is reduced by performing a first amplification, hereafter referred to as the Pre-selective amplification. A subset of all the fragments is amplified by using primers that are complementary to the adaptors with one additional nucleotide at the 3' end. Thus, pre-selective primers consist of a core sequence, an enzyme-specific sequence, and a selective single-base extension at the 3' end. These primers allow amplification only when a perfect match at both ends of the fragments occurs, that is, when bases flanking the restriction sites are complementary to the selective bases. After the pre-selective amplification there is a reduction of the complexity of the fragment pattern. Each primer contains a selective nucleotide of extension, so only one fragment out of 16 will be amplified on average, reducing the number of fragments.

Finally, the forth step comprises the second or Selective amplification. By conducting a second amplification the complexity of the fragment pattern is reduced even more. The complexity of the genomic DNA determines the number of amplified fragments as well as the choice of enzymes and the number and type of selective nucleotides in the PCR primers (Bleas et al 1998). The selective primers whose structure follows the same principle (a core sequence, an enzyme specific sequence and a selective extension) are identical to the pre-selective primers, plus one or more nucleotides at the 3' end. As the optimal length of these primers depends on the complexity of the genome they might contain three or more selective bases of extension.

The number of amplified fragments can be conveniently controlled by the number of nucleotides added to the 3' end of selective primers. If selective primers contain a two-base extension only one out of 256 fragments will be amplified, while if they have an extension of three bases, one out of 4096 fragments will be amplified and so on (Bonin

*et al.* 2005). A successful selective amplification will produce a characteristic fingerprinting of 20 to 150 fragments within a range of 50 to 500 base pairs (Bonin *et al.* 2005).

The AFLP technique was originally designed to detect and visualize amplified fragments by using radio-labeled primers and autoradiography (Vos *et al.* 1995), as silver staining and polyacrylamide gels were commonly used. Currently, such techniques have been replaced by a more reliable, safer, and faster method based on fluorescence. The fluorescence detection system allows automatic sequencers (e.g. Applied Biosystems (ABI) and MegaBACE) to measure the signal intensity (fluorescence) (e.g. Hartl and Seefelder 1998, Huang and Sun 1999) and the length of the AFLP fragments via capillary electrophoresis (Schwartz and Ulfelder 1992). By using capillary systems along with an internal size standard (DNA fragments of known size labeled in a different dye color) AFLP fragments can be sized with a resolution of a single-base pair (Wenz *et al.* 1998, Lindstedt *et al.* 2000). The capillary instruments detect fragments present in the wave length of a fluorophore (e.g. FAM, HEX), then an electronic profile comprising Relative Fluorescence Units (RFU) versus fragment size is generated. Following such principle, several selective primers (commonly EcoRI primer) could be labeled with different fluorophores (having different wavelength emissions), then products from different primer combinations might be pooled for capillary electrophoresis, performing a multiplexing process for each individual (Wenz *et al.* 1998, Meudt and Clarke 2007). AFLP profiles can be displayed as electropherograms by using specialized software packages. These software packages can determine the size of amplified fragments classifying them according to their size with a resolution of a base pair (Bonin *et al.* 2005). Polymorphisms are visualized as peaks present in some samples and absent in others.

## 3.2 Materials and Methods

### 3.2.1 Tissue samples

The study was conducted with approval of the Animal Ethics Committee of the University of Canterbury (permit Ref: 2012/24R).

Collection of samples was carried out in and around the 52 km<sup>2</sup> Ngel Nyaki and Kurmin Danko Forest Reserve (7°30'N, 11°30'E), between 1400-1600 masl, on the Mambilla Plateau in Nigeria's Taraba State. Tissue samples were collected during the rainy season of 2012 from July to October. Active searches and a combination of visual and acoustic techniques were conducted to locate animals during both the day and night. Adults and tadpoles were caught, toe or tail clipped and released at their capture point. Only one tissue sample per individual was taken. Tadpoles were caught using nets. Liver samples were taken post-mortem from five adult voucher specimens (two individuals of *C. schioetzi*, one of *L. bicolor*, and two individuals of *Astylosternus* spp.). Individuals were euthanized by immersion in an aqueous solution of Tricaine methanesulfonate (250 mg/liter), also known as MS-222, and preserved in 10% neutral-buffered formalin. Tissue samples were preserved in 95% Ethanol for subsequent genetic analysis. For each of the four anuran target species, 10 to 30 tissue samples were collected from several sites including forest and riparian forest fragments (Table 3-1). As mentioned before, such samples were used to conduct a population genetic study as well (see chapter 4).



Table 3-1. Number of samples per species per site. Main Forest I (MFI), Main Forest II (MFII), Main Forest III (MFIII), Main Forest IV (MFIV), Fragment 1 (F1), Fragment 2 (F2), Fragment 5 (F5), Fragment 6 (F6), Kurmin Danko 3 (KD3), Kurmin Danko 4 (KD4) (see Figure 4-1 in chapter 4).

	MFI	MFII	MFIII	MFIV	F1	F2	F5	F6	KD3	KD4	Total
<i>Cardioglossa schioetzi</i>	13				30	20	21				84
<i>Leptodactylodon bicolor</i>	31	20	24	30				26			131
<i>Astylosternus</i> sp. 1	8	29	7	20				25	22	28	139
<i>Astylosternus</i> sp. 2	17	1	18	6							42

Only one tissue sample per individual was taken.

### 3.2.2 Sample processing

#### 3.2.2.1 DNA extractions

Genomic DNA was extracted from tissue samples using a modified CTAB (Cetyltrimethylammonium bromide) protocol (Weising *et al.* 1995). Refer to Appendix I for the complete protocol. Briefly, tissue was incubated in CTAB isolation buffer, followed by chloroform–isoamyl alcohol extraction. An RNase treatment step was applied to remove RNA, afterwards, cold isopropanol was added and samples were left in a fridge (4°C) overnight to allow DNA precipitation. DNA was pelleted via centrifugation, then DNA pellets were washed with ethanol, dried, and dissolved in 10 mM Tris-HCl, 1 mM EDTA (TE) buffer. DNA samples were stored at -20°C.

The AFLP technique requires high-quality DNA, so the extraction phase is crucial for the success of the entire process. Restriction enzymes are highly sensitive to impurities (Weising *et al.* 2005) preventing the complete digestion of genomic DNA. Hence, using DNA extractions of outstanding quality ensures a successful template preparation for subsequent steps. A NanoDrop spectrophotometer (Thermo Scientific NanoDrop™

1000) was used to determine quality and concentration of DNA by measuring optical density (OD<sub>260</sub>). DNA of good quality is reported at a ratio of absorbance (260/280nm) between 1.8 and 2 (Wilfinger *et al.* 1997).

### **3.2.2.2 DNA preparation**

As no previous information on molecular markers has been generated for any of the four target species, it was critical to optimize the AFLP technique, so different tests, including the use of diluted or undiluted DNA templates, and trials of several primer combinations were performed to optimize the AFLP assays.

For the preparation of DNA two trials were performed: 1) double-digestion, using two restriction enzymes in the same reaction to digest the genomic DNA, then ligation was performed as an independent reaction, and 2) simultaneous digestion-ligation reaction, in which digestion of the genomic DNA and ligation with the adapters was done in one reaction. In each case EcoRI and MseI restriction enzymes were used. Best results were obtained by performing double-digestion of genomic DNA with ligation immediately after as an independent process.

Digestion was carried out using the two enzymes, EcoRI and MseI. The former has a restriction site of 6 base pairs and is a rare cutter, whereas the latter is a frequent cutter and consists of a 4-base pair restriction site (Table 3-2). This enzyme combination has proven to be efficient for the digestion of long size genomes (Bonin *et al.* 2005), making it ideal for the complex DNA genome of amphibians (e.g. Mila *et al.* 2010, Correa *et al.* 2012). Trials of digestion using this combination of restriction enzymes were conducted on all of the target species in this study.

Whole genomic DNA was digested to produce restriction fragments by using 1 unit of each of the restriction enzymes EcoRI (Roche) and MseI (New England Biolab), and 1x SuRE/Cut Buffer H (Roche). The 25µl final volume was incubated for 3hrs at 37°C. Subsequently, double-stranded adapters were ligated to the restricted fragments. Adaptor pairs were prepared by adding equal volumes of the individual synthetic oligonucleotides, EA2 + EA3 (10µM) for EcoRI, and MA1 + MA2 (10µM) for MseI (Table 3-2). The mixture was heated at 65 °C for 5 minutes and cooled at room temperature. Alternatively, the mix can be at room temperature for a period of 30 minutes or an hour to allow strands to anneal. A 20 µl mixture containing 5µl of digested DNA, EcoRI adapters (0.75µM), MseI adapters (1.5µM), 1 unit T4 DNA Ligase, and 1x T4 DNA Ligase Buffer (Thermo Scientific) was then incubated at 37°C for a period of 10 hrs. Digestion and ligation reactions were incubated in a thermocycler machine (Eppendorf thermocycler Mastercycler® ep gradient S) to keep a constant temperature through the process. After each of these reactions, samples were visualized in 1% TBE agarose gels to ensure complete digestion of genomic DNA and adequate ligation of adapters to the resulting restriction fragments. Trials using diluted restriction products (1:9) were conducted on the ligation reaction. The ligation mixture was developed under the same conditions as described above.

### **3.2.2.3 AFLP protocol**

AFLP profiles were obtained using two increasingly selective amplification processes following a protocol modified from Vos *et al.* (1995). The first amplification or the Pre-selective PCR was performed using 5µl of ligation product as the template DNA in a 20 µl reaction containing the pre-selective primers ENP (0.6µM) and MNP (0.6µM) (Table

3-2), 1x PCR buffer, Betaine (1M), MgCl<sub>2</sub> (2.5mM), dNTPs (0.2mM) and 1unit BioTaq DNA polymerase (BIOLINE). The amplification cycle profile was as follows: denaturation 30 s at 94°C, annealing 1 min at 56°C, and extension 1 min at 72°C for 25 cycles, plus two minutes of final extension at 72°C.

Table 3-2. Restriction enzymes, adapters, and primer sequences used during the AFLP assays.

Sequence (5'-3')		
Restriction enzymes	EcoRI	G <sup>^</sup> AATTC
	MseI	CTTAA <sup>^</sup> G
		T <sup>^</sup> TAA
		AAT <sup>^</sup> T
Adapters	EA2	CTCGTAGACTGCGTACC
	EA3	AATTGGTACGCAGTCTAC
	MA1	GACGATGAGTCCTGAG
	MA2	TACTCAGGACTCAT
Pre-selective primers	ENP	GACTGCGTACCAATT
	MNP	GATGAGTCCTGAGTAA

The quality of pre-selective products was examined by visualizing PCR products on 1% TBE agarose gels.

As mentioned before, the length of the primers and the combination of different pairs of primers are critical factors for the selective amplification stage as the complexity of the profiles relies on the addition of random nucleotides. Hence, a selective primer screen was conducted to optimize the number of amplified fragments that will produce clear and scorable AFLP profiles. Trials on the length of selective primers were performed by using oligonucleotides identical to pre-selective ones plus three and four bases of

extension at the 3' end. Tests on efficacy of different primer pairs were conducted using 16 different primer combinations (Table 3-3).

Selective PCR was conducted using 2 µl of pre-selective DNA template and a mixture (11 µl total volume) comprising selective primers ESP1B (0.45 µM) and MSP (1 µM, Table 3-3), 1x PCR Buffer, MgCl<sub>2</sub> (5 mM), dNTPs (0.25 mM), and 0.5 units of Amplitaq Gold DNA Polymerase (Applied Biosystems). The ESP1B selective primer was labeled with a fluorescent dye (6FAM, Applied Biosystems) for analysis on an automated sequencer. Selective amplification was performed following the touchdown program: initial incubation 10 min at 95°C to activate the Amplitaq Gold DNA Polymerase, 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C for one cycle. The annealing temperature was reduced by 0.7°C on each of the next 12 cycles, hence, in the first cycle the annealing temperature was 65°C, and it was reduced each cycle to 56°C after 12 cycles. This last annealing temperature was continued for 25 cycles and was then followed by a final extension period of 30 min at 72°C. The touchdown PCR promotes high stringency (high annealing temperature) not only eliminating faulty priming, but also ensuring highly specific amplification (Ajmone-Marsan *et al.* 1997, Mueller and Wolfenbarger 1999). Amplifications were performed using an Eppendorf thermocycler Mastercycler® ep gradient S.

Negative controls consisting of a reaction with only the reagents without template DNA were used in every step of the AFLP protocol to detect any exogenous contamination.

Table 3-3. Selective primer sequences with 3 and 4 nucleotides of extension. Sixteen combinations of selective primers (ESP1B plus one MSP primer) were tested to optimize the number of amplified fragments yielding clear and scorable profiles.

<b>Name Primer</b>	<b>Sequence</b>	<b>+ 3 bases</b>	<b>+ 4 bases</b>
ESP1B	GACTGCGTACCAATT	CAG	
MSP1	GATGAGTCCTGAGTAA	CAG	CAGA
MSP2	GATGAGTCCTGAGTAA	CTG	CTGC
MSP3	GATGAGTCCTGAGTAA	CGA	CGAT
MSP4	GATGAGTCCTGAGTAA	CAC	CACG
MSP5	GATGAGTCCTGAGTAA	TAG	TAGC
MSP6	GATGAGTCCTGAGTAA	CCT	CCTC
MSP7	GATGAGTCCTGAGTAA	GTC	GTCA
MSP8	GATGAGTCCTGAGTAA	CCC	CCCT

### 3.2.3 Detection and scoring of AFLP markers

The amplified products from selective amplification were detected on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Selective products (1µl) were added to a mixture of 0.125µl of Gene-Scan LIZ size standard (Applied Biosystems), and 10µl of Hi-Di formamide. The mixture was transferred to 96 well-plates, heated 5 min and cooled on ice before being loaded in the ABI analyzer.

AFLP fragments were analyzed using GENEMAPPER 4 (Applied Biosystems). Peaks were called using the default settings (sum-of-signal normalization, a bin width of 1 bp, maximum peak width of 1.5 bp, pull-up ratio of 0.1 and scan set to 1) except for the peak height detection which was set at 100 Relative Fluorescent Units (RFU).

Amplified fragments were displayed in electropherograms in which each peak corresponds to a fragment or locus (Figure 3-2). A peak was considered a locus as long as its intensity was no less than 100 RFU and its length was at least 100 bp. Thus, only peaks ranging between 100 and 300 base pairs (the limit of clear resolution of fragment detection as peaks above 300 bp had low signal), and with a minimum of 100 RFU were included in the analysis. The sizing data for each profile was checked manually and modified when necessary. The agreement of the size standard peaks and the samples was reviewed in order to detect deviations in the peak positions. It was necessary to check that all peaks were correctly identified by the software, checking the sequential order of the labels. Modification of the size standard definition was applied to correct miscalled peaks when the size standard fragments did not match the positions of the detected peaks. The presence or absence of all fragments was confirmed manually. As suggested by Stölting *et al.* (2011), markers were scored for all the individuals in the same analysis session to prevent scoring errors when analyzing several groups of samples.

In order to assess the informativeness of the primer combinations each AFLP marker was measured using the Polymorphism Information Content (PIC) which provides an estimate of the discriminating power of the marker. PIC refers to the value of a marker for detecting polymorphism within a population, and depends on the number of detectable alleles and the distribution of their frequency. It was calculated as proposed by Roldán-Ruiz *et al.* (2000) as  $PIC_i = 2f_i(1 - f_i)$ , where where  $PIC_i$  is the

polymorphic information content of marker ' $i$ ',  $f_i$  is the frequency of the fragment present in the data set. PIC was then averaged over the fragments for each primer combination. For dominant markers PIC is a maximum of 0.5 for  $f=0.5$ .

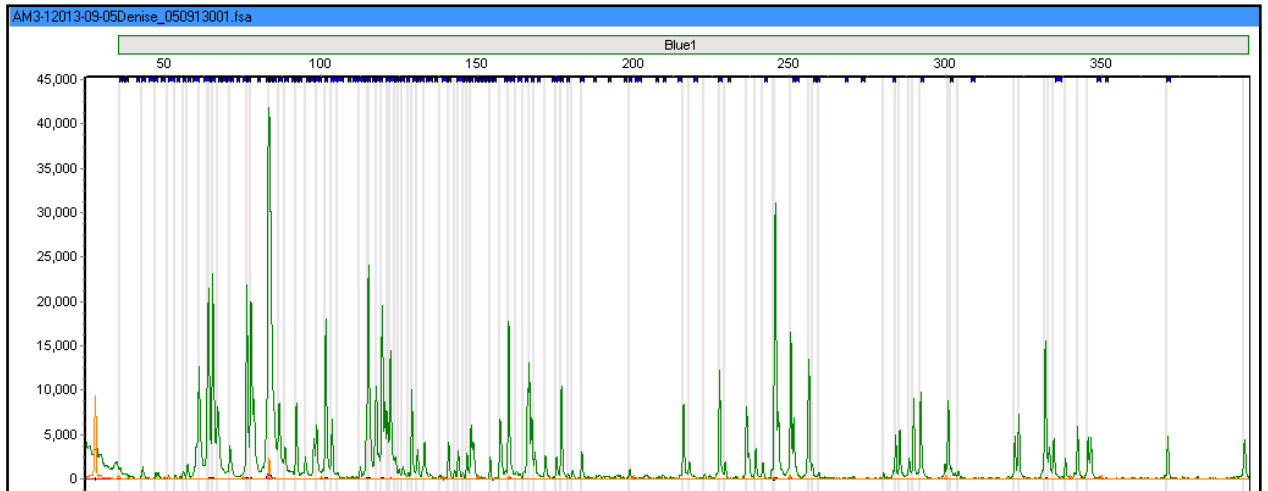


Figure 3-2. Example of an AFLP profile displayed as an electropherogram. Each peak represents an amplified fragment (x-axis size of the peaks in base pairs, y-axis height in RFU).

### 3.2.4 Reproducibility and genotyping error

Reproducibility tests were conducted by replicating samples. As suggested by Bonin *et al.* (2004) and Meudt and Clarke (2007), a subset of the samples (ideally 5-10% of the total number of samples) is recommended to be replicated randomly to detect fragments producing erratic patterns. Thus, 19 samples (5%) were chosen randomly for each of the preferred primer combinations to be duplicated.

Genotyping errors are described as the mismatches between two or more genotypes obtained on different occasions from the same sample, thus there is no correspondence between the real genotype and its replicates. As genotyping errors might be present in any genetic data set, their quantification is important to detect unreliable markers (unstable or difficult to score), straighten up the data and prevent misinterpretation of



the results (Bonin *et al.* 2004, Pompanon *et al.* 2005). Then, comparing the binary matrices of replicated samples, the genotyping error rate per locus was calculated as the ratio between the number of single-locus mismatches (*ml*), and the number of replicated loci (*nt*) (Pompanon *et al.* 2005). In order to limit genotyping errors several measures were implemented throughout the AFLP process as recommended by Bonin *et al.* (2004). For instance, negative controls (using only the reagents not template DNA) were used in every step of the AFLP process (digestion, ligation, pre-selective and selective amplifications) to detect any exogenous contamination. A significant quality control was carried out by running on agarose gels the products from each of the steps. As this technique involves non taxon-specific amplification (that is, any DNA present in the reaction would generate fragments different of the desired product), negative controls and product visualization were critical to produce reliable and high quality profiles. Other measures contributing towards keeping the error rate to a minimum were also adopted: such measures included use of the same thermocycler device, performing independent reproducibility tests, checking data manually and quantify the genotyping error rate. A few problematic samples were discarded for the characterization analysis as after running them twice the profiles were still unable to be scored.

### **3.3 Results**

Extraction following the CTAB protocol produced high-quality DNA. The Nano Drop 1000 spectrophotometer was used to determine quality and concentration of DNA. To evaluate the purity of DNA the ratio of absorbance at 260 and 280 nm was recorded. A ratio of ~1.8 – 2.0 is reported when DNA is acceptable or good quality. For the four target species DNA extractions were successful using the CTAB protocol obtaining

concentrations from 2.56 - 3330.86 ng/μl. The 260/280 absorbance ratio ranged from 1.41 to 2.5, and 89% of the extractions had ratios between 1.8 and 2 which is the range of high quality DNA. In addition, 1% TBE agarose gels were run to visually confirm quality and no degradation of DNA was observed (Appendix II). A single, well defined, high molecular weight band was observed for each of the DNA extractions.

As DNA of high quality is one of the most important requirements to get outstanding AFLP profiles, the entire AFLP process was performed for sample subsets immediately after the extraction of the DNA. By following a continuous and strict work flow, not only was the degradation of genomic DNA prevented, but also degradation of DNA templates required at each of the stages, particularly the ligation products that are highly susceptible to deterioration (see Appendix II).

### **3.3.1 DNA preparation and AFLP profiles**

Trials for digestion-ligation in the same reaction did not produce fragments. Usually in AFLP assays, restriction and ligation can take place in a single reaction, however, for the present study preparation of DNA was successfully completed by performing digestion and ligation in two successive steps.

The combination of the restriction enzymes EcoRI and MseI worked well on the target species. Products from digestion were visualized on agarose gels to confirm the complete digestion of the genomic DNA. Total digestion was observed as 'smear bands' denoting that a number of restriction fragments were produced (Figure 3-3).

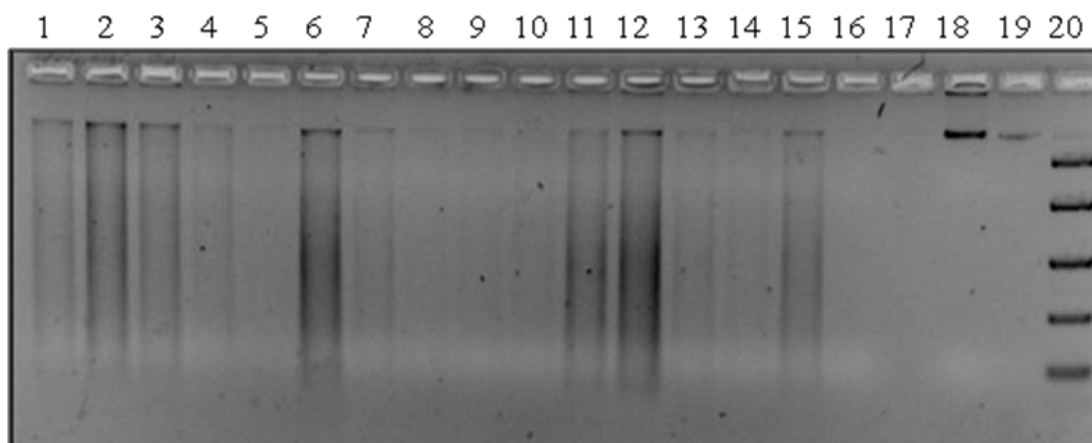


Figure 3-3. Products of the double-digestion using EcoRI and MseI restriction enzymes. Lanes 1-15 digested DNA. Lane 16, negative control. Lane 17 empty. Lanes 18 and 19, undigested DNA. Lane 20, Easy ladder marker (100-2000bp). Digested DNA was visualized on 1% TBE agarose gel, 95Volts for 30 minutes.

Like digestion, ligation products were run on agarose gels to evaluate their quality and to verify that no bands were present in the negative controls (Figure 3-4).

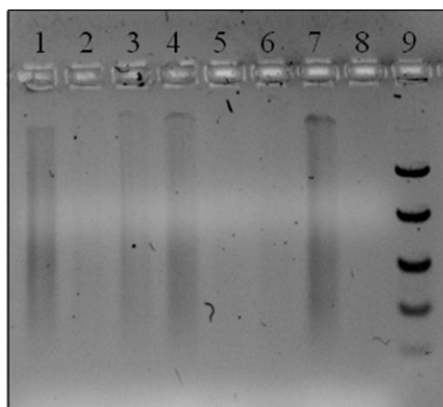


Figure 3-4. Ligation of adapters to restriction fragments in lanes 1 to 7. Lane 8, negative control. Lane 9, Easy ladder (100-2000bp). When successfully ligated, fragments on the gel look like a smear across the lane; for some samples only a faint smear is visualized (lanes 2, 5, and 6). Products of ligation were visualized on 1% TBE agarose gels, run at 95 volts for 30 minutes.

No difference was recorded when diluted and undiluted digestion products were used as templates. Smear bands of similar intensity were visualized on agarose gels for ligation products generated with diluted and undiluted DNA.

Likewise, no notable differences were observed in peak patterns when electropherograms of profiles generated using undiluted and diluted DNA were compared, however, some peaks showed a slightly lower intensity with diluted DNA.

High concentrations of undiluted DNA templates did not influence the performance of the AFLP assay affecting final results or profiles, therefore, ligation, pre-selective and selective amplifications were implemented by using undiluted DNA.

### 3.3.2 Pre-selective and Selective PCR

Agarose gels following this first amplification were used to ensure successful reactions and high quality bands to be used in subsequent amplifications, as well as to detect any event of contamination by using negative controls. Fragments were visualized as smear bands between 200 to 600bp and were uniform across samples (Figure 3-5).

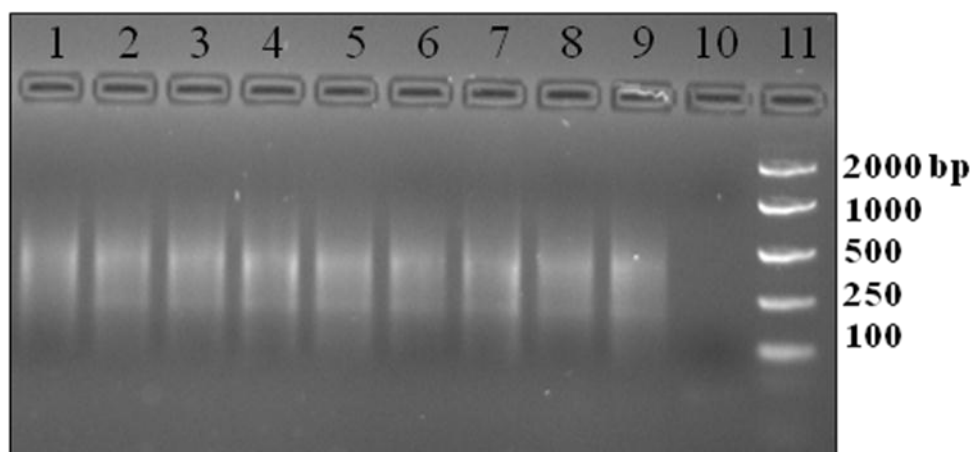


Figure 3-5. Quality and size range of pre-selective products. Fragments generated by pre-selective PCR were visualized on 1% TBE agarose gels, run for 40 minutes at 95volts. Lanes 1 to 9 depict good quality PCR products, as the smear bands are between 200 and 600bp and occur uniformly across samples. Lane 10, negative control. Easy ladder marker, lane 11.

Selective amplification assays were optimized by testing primers of different length or number of nucleotides of extension. Sixteen selective primer combinations were screened and two were selected for the analysis. The 14 primers not selected yielded no amplification products, contained poorly resolved peaks or highly complex patterns of peaks. Trials of several primers with three selective bases produced very intricate profiles. However, by adding one more nucleotide, that is, four selective nucleotides, a clear reduction of the complexity of fragment patterns was observed as the number of

peaks was reduced substantially. Figure 3-6 illustrates two AFLP profiles generated by using MseI selective primers with three and four nucleotides of extension, respectively. The preferred two pairs of selective primers, ESP1B / MSP3, and ESP1B / MSP6 produced reliable peak patterns. The EcoRI selective primer, ESP1B, contained three selective bases, whereas MSP3 and MSP6 (MseI selective primers) contained four bases of extension each (Table 3-4).

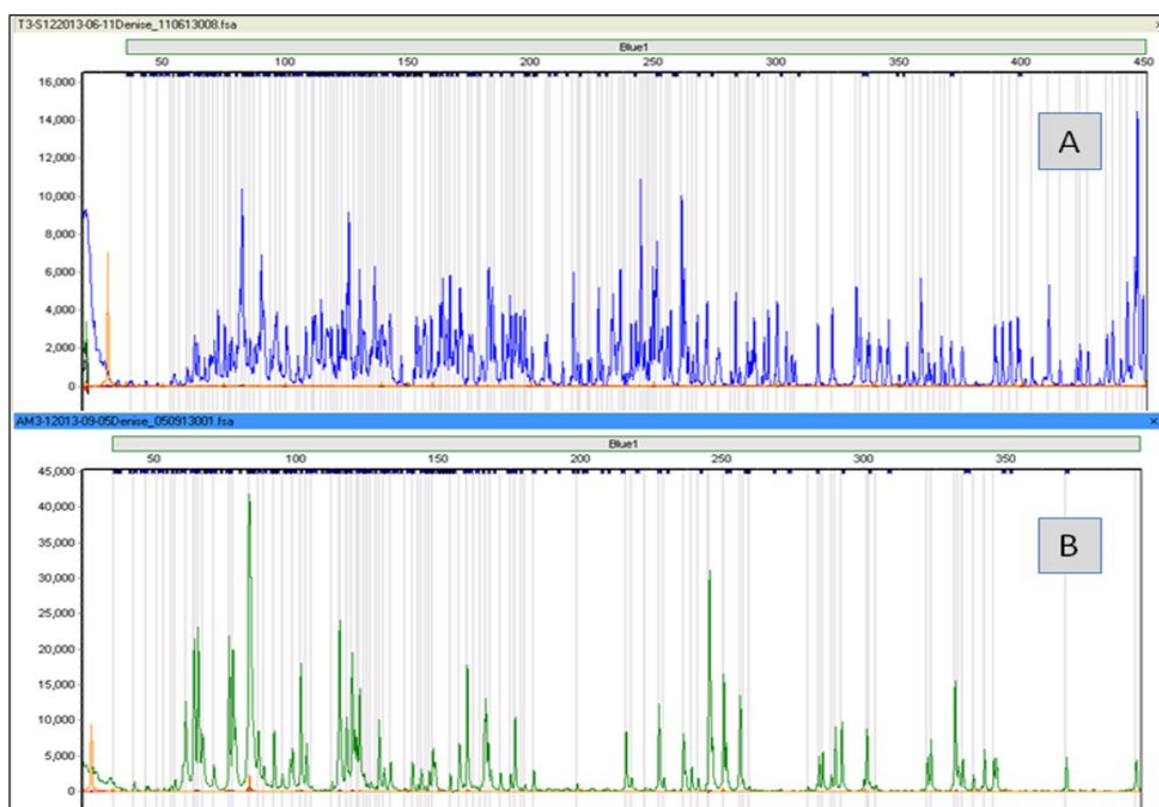


Figure 3-6. Test of selective primers of different length. A) Using both EcoRI (FAM) and MseI primers with three selective bases. B) Using EcoRI (VIC) and MseI selective primers with three and four bases of extension respectively.

Table 3-4. Sequences of the selective primers that produced reliable fragment patterns.

	Sequence (5'-3')	5' Modification
ESP1B	GACTGCGTACCAATTCAG	fluorescent-labeled, 6FAM
MSP3	GATGAGTCCTGAGTAACGAT	
MSP6	GATGAGTCCTGAGTAACCTC	

A total of 631 peaks were produced with sizes ranging from 50 to 300 bp from the two preferred primer pair combinations. Of the total fragments scored 554 were polymorphic (87.7%) (Table 3-5).

Table 3-5. Combination of primers used to generate AFLP profiles for all the target species.

<b>Primer combination</b>	<b>Total Number Loci</b>	<b>Polymorphic Loci</b>	<b>% polymorphism</b>	<b>Range bp</b>
ESP1B / MSP3	272	238	87.5	100-300
ESP1B / MSP6	359	316	88	100-299
Total	631	554	87.7	

The minimum number of polymorphic loci in a single species was 51 for *Leptodactylodon bicolor* with the ESP1B/MSP3 primer pair, and the maximum number of scored polymorphic loci was 108 for the ESP1B/MSP6 combination in *Astylosternus* sp. 1 (Table 3-6). The average polymorphic loci per primer combination per species was 59.5 for ESP1B/MSP3 and 79 for ESP1B/MSP6.

Marker informativeness for the two preferred pairs of primers was analyzed using the PIC parameter (Table 3-7). The lowest (0.211) and highest (0.283) PIC values were observed for the ESP1B/MSP3 combination. The average values were 0.247 (SD =

0.030) and 0.264 (SD = 0.009) for ESP1B/MSP3 and ESP1B/MSP6 respectively. No differences were detected between the two primer combinations ( $F(1,6) = 1.13$ ,  $P = 0.332$ ).

Table 3-6. Combination of primers used for the analysis and variation in loci for each species.

	<b>Total number loci</b>	<b>Polymorphic loci</b>	<b>Range bp</b>
<b><i>Cardioglossa schioetzi</i></b>			
ESP1B / MSP3	56	53	100-259
ESP1B / MSP6	81	72	100-291
Total	137	125	
<b><i>Leptodactylodon bicolor</i></b>			
ESP1B / MSP3	64	51	100-284
ESP1B / MSP6	74	67	100-291
Total	138	118	
<b><i>Astylosternus</i> sp. 1</b>			
ESP1B / MSP3	94	83	100-300
ESP1B / MSP6	120	108	100-299
Total	214	191	
<b><i>Astylosternus</i> sp. 2</b>			
ESP1B / MSP3	58	51	101-275
ESP1B / MSP6	84	69	100-268
Total	142	120	

Table 3-7. PIC values for each AFLP primer combination per species.

	ESP1B/MSP3	ESP1B/MSP6
<i>Cardioglossa schioetzi</i>	0.258	0.257
<i>Leptodactylodon bicolor</i>	0.211	0.269
<i>Astylosternus</i> sp. 1	0.238	0.256
<i>Astylosternus</i> sp. 2	0.283	0.275
Average	0.247	0.264

### 3.3.3 Reproducibility and genotyping error

The reliability of the AFLP protocol and the markers was tested by using replicated samples (Figure 3-7). The error rate per locus was calculated from replicated AFLP profiles. Nineteen profiles for each of the preferred primer combinations were randomly chosen for duplication. The intensity of some of the duplicates was too low making them unreliable to score and to be used for calculating the error rate, so 15 AFLP profiles of the ESP1B/MP3 and 16 of the ESP1B/MSP6 primer combinations were used for this purpose. The estimation of the genotyping error was conducted after scoring by comparing the presence-absence matrices of such profiles. The estimated genotyping error per locus was 5.5% (SD = 5.3) for ESP1B/MSP3 and 7.1% (SD = 5.6) for ESP1B/MSP6. The average rate was 6.3% and was obtained by taking into account the two pairs of primers for which the average number of mismatches per profile was 6.7.



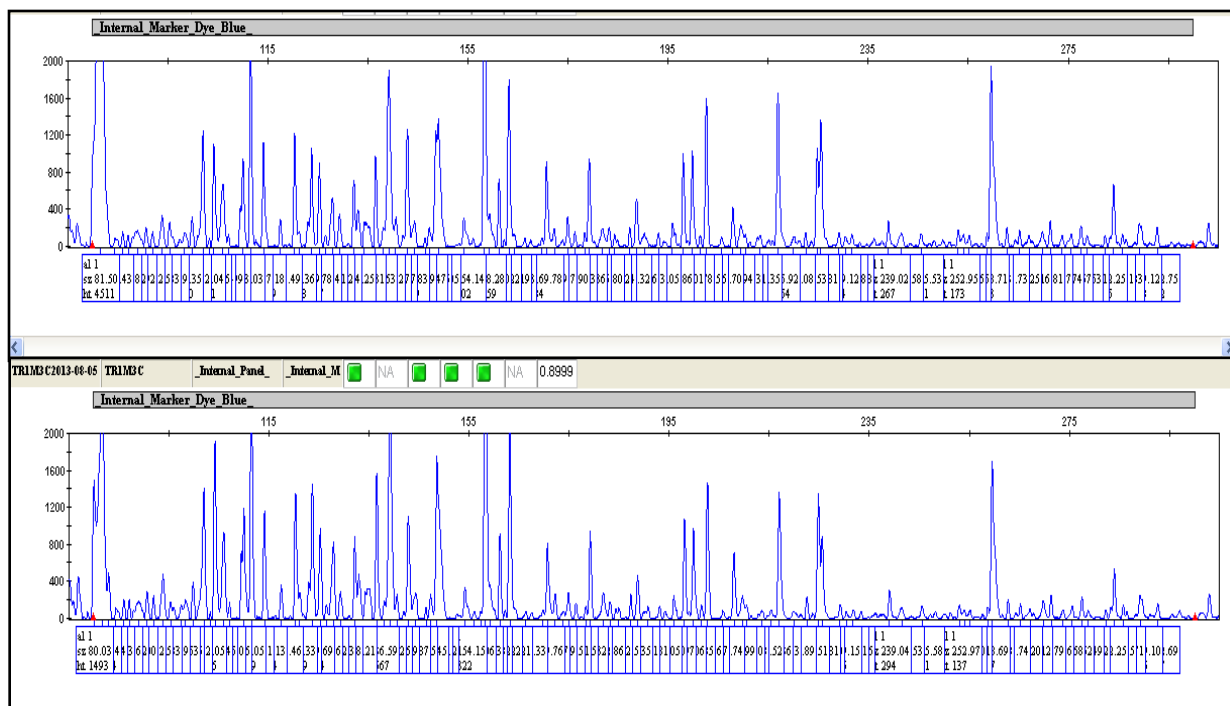


Figure 3-7. Example of repeatability tests using replicates. A consistent pattern of fragments was obtained from independent AFLP assays of the same individual.

### 3.4 Discussion

Following a modified AFLP protocol of Vos *et al.* (1995), AFLP markers were generated for four frog species for which no species-specific molecular markers have been isolated. Results provided evidence supporting AFLP as a useful technique capable of being applied to any samples of DNA and that the complexity of the fragment pattern was reduced by adding selective nucleotides to the primers used for the second amplification. Findings here support the AFLP technique as a suitable option when no prior or specific molecular markers are available. This is true for all four focus species as they are poorly known and no molecular markers such as microsatellites have been yet isolated for any member within the Arthroleptidae family.

As suggested in the literature (Bleas *et al.* 1998, Bensch and Åkesson 2005), high DNA quality was one of the most important factors to yield reliable and scorable AFLP fingerprints. Good quality DNA was obtained for the four target species with the CTAB protocol. Negative controls, that is, using only the reagents without template DNA, were applied for every step of the AFLP protocol to detect exogenous contamination but also to minimize genotyping errors. Another routine precaution taken during the process was the visualization on agarose gels of the products from each of these steps. It was a significant measure to prevent or identify the generation of fragments other than desired as the AFLP technique involves amplification of non taxon-specific DNA. Both negative controls and agarose gels were critical to produce reliable and high quality profiles. Other measures, for instance, the use of the same thermocycler device, tests of reproducibility, and error rate quantification contributed to keep the genotyping error as low as possible.

For AFLP, it has been stated that a typical genotyping error rate is between 2 and 5% (Bonin *et al.* 2004, Bonin *et al.* 2007). The average genotyping error rate per locus reported here (6.3%) was higher than other AFLP reproducibility tests in plants (Hasbún *et al.* 2012) or animals (Ajmone-Marsan *et al.* 1997). The error rate estimation was also higher than others reported in amphibian studies. For instance, Mila *et al.* (2010) reported an average error rate as low as of 0.8% in the Pyrenean brook newt *Calotriton asper*. Even though precautions were taken, genotyping errors could be generated at every step of the AFLP protocol followed in this study. When electropherograms of duplicated profiles were overlapped it was observed that most of the mismatches were a slight shift between two peaks, leading to a false absence of a given locus when binary matrices were compared. This type of error might be reduced during the normalization

process (Bonin *et al.* 2004) and by checking manually the overlapped electropherograms.

The general genotyping error presented here was slightly higher than the typical or acceptable range. The error rate was kept as low as possible by modifying the criteria of selection of fragments used for the analysis. Originally, the fragments from 50 to 300 bp were included in the analysis, however, after performing the test of reproducibility and estimating the genotyping error it was observed that most of the mismatches occurred within the first 50 bp of the duplicated profiles and an extremely high error rate was recorded. Therefore, it was decided to take into account only fragments between 100 and 300 bp, and as a consequence, the loci used for the assessment of the AFLP markers were reduced in number but no loss of genetic signal was detected. In this manner, the overall error rate was minimized without compromising the population genetic signal.

It has been observed that important population genetic signals (Crawford *et al.* 2011, Zhang and Hare 2012) as well as phylogenetic resolution (Holland *et al.* 2008) are sometimes lost by excluding loci to obtain acceptable error levels. Tradeoffs between selecting the loci with lowest error rates to minimize overall genotyping error versus the potential for increasing population genetic signal by retaining more loci have been evaluated (e.g. Zhang and Hare 2012). Crawford *et al.* (2011) demonstrated that when removing questionable peaks from the analysis, a decrease in the error rate was observed. Their strict approach (thresholds of 100 RFU and singleton loci removed) retained less loci for the genetic analyses, and produced a strong signal of population structure.

While errors in genotyping data have been shown to have significant effects on parentage studies leading for example to a false paternity or maternity exclusion (Wang

2010), or masking the true segregation of alleles in linkage and association studies (Pompanon *et al.* 2005), in population genetic analyses (based on allele frequencies) the effect of error rates might be less severe (Pompanon *et al.* 2005, Dewoody *et al.* 2006). Thus, it is expected that the error rates of the two evaluated markers (ESP1B/MSP3 and ESP1B/MSP6) have a minor impact on the population genetics study for which they were employed (see chapter 4).

As the efficiency of a molecular marker technique depends on the amount of polymorphism detected, it might be argued that good quality markers were obtained with the preferred primer combinations (ESP1B/MSP3 and ESP1B/MSP6) as the total percentage of polymorphism was 87.7% (554 polymorphic loci in total). The level of polymorphism detected by AFLP in the present study is comparable to other studies using AFLP markers on amphibians (e.g. Curtis and Taylor 2003, Rogell *et al.* 2010, Fontenot *et al.* 2011). Moreover, PIC values showed that the two primer combinations were informative. Although there is no previous information for the target species to compare with, Sathyanarayana *et al.* (2011) assessed the AFLP method in legumes from the genus *Mucuna* and obtained PIC values ranging from 0.138 to 0.209, concluding that evaluated markers had high discrimination power. The PIC values for the markers reported here (see Table 3-7) have more discrimination power (ranging from 0.211 to 0.283) than those reported by Sathyanarayana *et al.* (2011).

Due to the increasing pressure generated by anthropogenic disturbances on the Mambilla Plateau, rapid conservation action is required for preserving amphibian species. Genetic information is now essential in the conservation design and decision making processes. As accurate and reproducible data is critical for conservation planning, the AFLP technique has proved to be a quick and reliable method able to

generate many loci at a rapid rate and at a relatively low cost. In the case of the amphibians on the Mambilla Plateau for which immediate conservation action is required, AFLP markers represent the best option as a comprehensive compilation of genetic information could be rapidly achieved using such a molecular marker system.

The assessment and optimization of AFLP markers provided baseline data to conduct a population genetic study involving the four target species. Chapter four comprises a study of amphibian population connectivity in both fragmented and continuous populations in and around Ngel Nyaki and Kurmin Danko Forest Reserve on the Mambilla Plateau, using the two preferred combinations of primers ESP1B/MSP3 and ESP1B/MSP6.

## Chapter 4

### **The genetic structure of four frog species of the Arthroleptidae from fragmented and continuous montane habitat in Ngel Nyaki and Kurmin Danko Forest Reserve**

#### **Abstract**

This study represents the first attempt to describe the genetic diversity and population genetic structure of species within the Arthroleptidae in a fragmented landscape. The genetic population structure of four frog species (*Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1 *Astylosternus* sp. 2) differing in geographic distributions in and around Ngel Nyaki and Kurmin Danko Forest Reserve was assessed. Amplified Fragment Length Polymorphism (AFLPs) were used to understand dispersal and connectivity among continuous and fragmented amphibian populations of target species. Two preferred primer combinations ESP1B/MSP3 and ESP1B/MSP6 yielded 631 loci, and 554 of these (87.7%) were polymorphic. Genetic diversity estimates were similar across populations within each of the four species, but significant differences were observed between the average heterozygosities of the four species ( $F(3) = 6.691$ ,  $P = 0.004$ ). The average heterozygosities ranged from 0.219 (SE = 0.008) observed in *C. schioetzi* to 0.170 (SE = 0.005) observed in *A.sp1*. *Cardioglossa*

*schioetzi*, *L. bicolor*, *A.sp.* 1 showed genetic differentiation between the forest and the riparian fragment populations. On the other hand, no significant genetic differentiation was detected among the populations located in continuous forest for any of the four frog species. Geographic and genetic distances were not significantly correlated for any of the four target species, suggesting no isolation by distance at this fine geographic scale.

## **4.1 Introduction**

### **4.1.1 Threats to the Nigerian montane forest**

Nigeria has the fastest growing human population in Africa (United Nations [UN], 2015) which has resulted in massive loss of native biodiversity through the loss of natural habitats and their transformation into urban and agricultural areas. This is true for the Mambilla Plateau, where the unique montane forest habitat and its associated flora and fauna are threatened by human and agricultural settlements, overgrazing and other anthropogenic activities (Chapman *et al.* 2004). The mountains of eastern Nigeria are part of the Cameroon Volcanic Line (Marzoli *et al.* 2000) and regionally significant in terms of endemism (Stuart *et al.* 2004). The mountains of the Mambilla Plateau in Taraba State are part of the Cameroon Highlands Forest ecoregion as identified according to the WWF Global 200 ecosystem classification (Olson and Dinerstein 1998) where the flora and fauna are among of the most diverse on the continent (Chapman and Chapman 2001, Burgess *et al.* 2004).

The forests within Ngel Nyaki and Kurmin Danko Forest Reserve the focus of the study presented here, are threatened by the Fulani herding their cattle into the forest, and by their annual burning of grasslands along the forest perimeter. Small, narrow riparian

forests outside of the reserve are extremely negatively impacted by these activities; the streams they fringe are used as watering holes and cattle regularly cross them which compacts and erodes the soil as well as causing forest fragmentation.

#### **4.1.2 Amphibians and habitat fragmentation**

Amphibians are the vertebrate group with the highest rate of species threatened with extinction (Stuart *et al.* 2004, Beebee and Griffiths 2005). Decline of amphibian populations has been observed worldwide; however, not all species have been affected equally. According to recent research on global amphibian decline, there are particular situations in which the loss of populations is more likely to occur. For instance, amphibian declines from regions of high altitude (Houlahan *et al.* 2000, Lips *et al.* 2003) or from areas being degraded and fragmented (Curtis and Taylor 2003, Cushman 2006) have been reported as the most vulnerable.

In general terms, habitat fragmentation has been recognized as one of the drivers of extinction of biodiversity, and has been stated as the main reason for amphibian population declines (Bowne and Bowers 2004). While amphibians are an integral component of both terrestrial and aquatic environments (Semlitsch 2003) they have a relatively narrow moisture and temperature tolerance and a high sensitivity to vegetation structure. Moreover some species have extremely specific habitat requirements for reproduction (Duellman and Trueb 1986, Semlitsch 2003). These and other factors make amphibians especially vulnerable to habitat fragmentation, urbanization, and other types of anthropogenic disturbance (Cushman 2006, Tsuji *et al.* 2011, Hale *et al.* 2012).



With regard to the genetic effects, habitat fragmentation is predicted to have considerable long-term effects on the genetic and demographic viability of populations. This is due to the combined effects of reduced population size and increased isolation (Young and Clarke 2000). Consequences of isolation among patches include: reduced dispersal and gene flow, increased levels of inbreeding, smaller effective population sizes and loss of genetic variation (Lacy and Lindenmayer 1995, Frankham *et al.* 2002). These genetic effects may compromise the fitness of populations and their ability to adapt to a changing environment, increasing the risk of extinction (Frankham 2005).

#### **4.1.3 Amphibian dispersal**

Dispersal resulting in gene flow is a critical process in ecological and evolutionary terms. For example it tends to homogenize population structure, promote the persistence of small populations and spread adaptive traits in changing environments (Slatkin 1987, Templeton *et al.* 2001, Manel *et al.* 2003). As recognized by a number of studies, dispersal plays a critical role in the ecology and biology of many species of amphibians (Nathan 2001, Baguette *et al.* 2013). Along with extinction-recolonization dynamics, dispersal is critical in the genetic structuring of amphibian populations (Berven and Grudzien 1990, SjöGren 1991); dispersers affect gene flow patterns, colonize new breeding habitats and recolonize habitats following extinction (Hanski 1994). Nonetheless, amphibians are frequently recognized to have poor dispersal abilities and high site fidelity (Berven and Grudzien 1990, Blaustein *et al.* 1994, Beebee 1996). Based on a literature review on spatially structured populations, Bowne and Bowers (2004) found that among vertebrates amphibians had the lowest movement rates among habitat patches. Dispersal can be estimated directly or indirectly. On the one

hand, estimating dispersal in the field, for instance by applying mark-recapture techniques, can be limited in space and time (Bohonak 1999) as it is only inferring the gene flow occurring at the time the observations are made (Slatkin 1987). On the other hand, using indirect methods by means of DNA markers to calculate allele frequencies, the genetic structure of a given species reflects dispersal over many generations and across populations. Dispersal and gene flow are significant processes that can largely impact populations in terms of demography, evolution and viability, which are crucial characteristics to ecologists, evolutionary biologists and conservation scientists (Richardson 2012).

Population connectivity is crucial to many ecological processes (Cushman *et al.* 2012). Habitat fragmentation affects distribution of populations (Hanski 2000), and dispersal patterns (Gibbs 1998, Baguette *et al.* 2013). In addition, fragmentation promotes inbreeding due to an accumulation of related individuals within isolated fragments, and decreases the genetic diversity due to genetic drift (Keyghobadi 2007, Allentoft and O'Brien 2010). These effects might be mitigated with sustained levels of dispersal between fragments (Cushman 2006), thereby reducing the probability of local extinction (Gibbs 1998). It has been found that small mammals (Fahrig and Merriam 1985, Henein and Merriam 1990) and amphibians (Angelone *et al.* 2011) in heterogeneous environments might be able to disperse through corridors which connect habitat fragments. As connectivity through corridor habitats among isolated populations can mitigate the negative effects of fragmentation it is critical to know if habitat fragmentation on Ngel Nyaki and Kurmin Danko actually prevents or limits dispersal of the four focal species. An understanding of animal movements is critical to develop effective conservation strategies (Semlitsch 2003). This information will be of utmost

importance because it will inform better decisions for amphibian conservation management in a biologically remarkable area such as the Mambilla Plateau.

#### **4.1.4 AFLP and genetic population structure**

One of the most widely used molecular markers to study the genetic structure of natural populations is Amplified Fragment Length Polymorphism (AFLP). The AFLP method is based on PCR amplification of restriction fragments of digested DNA. It provides fast and easily developed markers that should be positioned throughout the genome in any organism (Vos *et al.* 1995). However, as with any dominant markers, only the presence or absence of a DNA fragment can be detected at a given locus, thus, no heterozygous individuals can be recognized (Bensch and Åkesson 2005). The presence of a given fragment could indicate either homozygosity for the present allele or heterozygosity carrying both a present and an absent allele.

Previous studies in amphibians using AFLP suggest that this is an effective and reliable technique for genetic inference at a population level. AFLP markers have been used to evaluate dispersal rates and patterns of gene flow among newt populations (Mila *et al.* 2010), as well as the degree of genetic differentiation among fragmented populations of frogs (Measey *et al.* 2007) and salamanders (Curtis and Taylor 2003, Lucas *et al.* 2009).

The genetic population structure of four frog species (*Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1 *Astylosternus* sp. 2) within Arthroleptidae, the target amphibian family of the present study, with differing geographic distributions in the study area was assessed to understand dispersal and connectivity among

continuous and fragmented amphibian populations in and around Ngel Nyaki and Kurmin Danko Forest Reserve on the Mambilla Plateau. Specifically, it was necessary to know whether dispersal exists in continuous (among populations within the forest), and in fragmented habitat (between riparian fragment populations, but also between forest and riparian fragment populations).

#### **4.1.5 Species of interest**

The four frog species *Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1, and *Astylosternus* sp. 2, all of them members of the Arthroleptidae family, were selected to conduct the population genetics study presented here. The herpetological inventory recently developed in Ngel Nyaki and Kurmin Danko (See Chapter 2) provided important findings on the biology and ecology of these frog species.

*Cardioglossa schioetzi* was recorded most often near streams in riparian forests outside the Ngel Nyaki Forest Reserve boundaries. A few specimens, including tadpoles, were caught at the edge of the forest near the field station of the Nigerian Montane Forest Project. Only a few males (not enough to complete an acceptable /outstanding sample size for a population genetics study) were recorded calling in three different riparian forests on Kurmin Danko. *Leptodactylodon bicolor* as well as *A.* sp. 1 shared the same microhabitat, they were found together along the streams in the forest of Ngel Nyaki. *Astylosternus* sp. 1, was common and the most widely distributed frog within both the main forest and riparian forest fragments of both Ngel Nyaki and Kurmin Danko forest reserve. In contrast *A.* sp. 2 was collected only from the main forest in Ngel Nyaki Forest Reserve. Interestingly, *L. bicolor* was found in only one riparian forest outside of

the reserve, and no individuals were heard in Kurmin Danko forest reserve (see Figure 4-1).

*Cardioglossa schioetzi* has been listed as Endangered, whereas *Leptodactylodon bicolor*, *Astylosternus diadematus* and *A. rheophilus* are listed as Vulnerable on the IUCN Red List of Threatened Species ([www.iucnredlist.org](http://www.iucnredlist.org)). A common denominator for cataloguing these species on the IUCN Red List is their severely fragmented distribution, and the declining extent of their habitat in the montane forests of Cameroon and Nigeria. In addition, there is no information available on their population status, and they are generally poorly known species.

Little is known of the biology and ecology of the four focus species, however, it should be noted that *Astylosternus diadematus* is polyploid (Bogart and Tandy 1981). No reports or studies on the population genetic structure have been published for any of the four species.

In this study, the following questions have been addressed for each of the four target species:

1. What is the level of genetic variability within their populations?
2. How is genetic diversity distributed within and among populations?
3. How are their populations related?
4. Is there a geographical relationship between populations?
5. Is gene flow occurring between forest and riparian fragment populations, or between populations in the same continuous forest?

## 4.2 Hypothesis and predictions

Given the information mentioned earlier, it is expected that some gene flow will be observed among populations within the forests but gene flow is unlikely to occur between the forest and the riparian forest fragments (and *vice versa*) for three of the target species. That is, for the species *Cardioglossa schioetzi*, *Leptodactylodon bicolor* and *Astylosternus* sp. 1 which have populations in both forest and riparian forests fragments, little or no gene flow is expected between the forest and fragments in any direction. Therefore the hypothesis is that high values of genetic differentiation would be observed among forest and riparian fragment populations. Due to its relatively high density and wide distribution in both forest and riparian fragment forests in Ngel Nyaki and Kurmin Danko, *A. sp. 1* is hypothesized to be the species least affected by fragmentation; theoretically a prediction would be for relatively high levels of genetic diversity and low genetic differentiation among populations within the forest. Likewise, for *A. sp. 2*, recorded only in the forest, low levels of genetic differentiation among populations would be predicted. In contrast, due to its constricted geographical distribution in Ngel Nyaki Forest Reserve, *C. schioetzi* might be the species most affected by fragmentation. A strong genetic structure could be observed as the riparian forests outside of the reserve have been severely fragmented by crossing cattle and the streams are negatively impacted by human activities. As for *L. bicolor*, high levels of genetic diversity among forest populations, and absence of gene flow between the riparian fragment and the forest populations are expected.

## 4.3 Material and methods

### 4.3.1 Study area and field sampling

The study was conducted in and around the 46 km<sup>2</sup> Ngel Nyaki and Kurmin Danko Forest Reserve (7°30'N, 11°30'E). While developing the herpetological inventory (Chapter 2) four key species of amphibians were selected (see above). As stated before in chapter 2, from July to October of 2012 tissue samples (clipped toes for adults and tail for tadpoles) were collected for the four target species in different sites including forest and riparian forest fragments (Figure 4-1). Although tissue samples of the four frog species were collected from several riparian forest fragments as well as sites within the forest in both Ngel Nyaki and Kurmin Danko, only 10 sites produced at least 10 tissue samples for any target species. Sites with less than 10 collected tissue samples (for any of the target species) were not used for this study. With regard to the continuous forest four sites were sampled within the Ngel Nyaki Forest Reserve: Main Forest I (MFI), Main Forest II (MFII), Main Forest III (MFIII), and Main Forest IV (MFIV). Outside of the reserve the four riparian forest fragments, Fragment 1 (F1), Fragment 2 (F2), Fragment 5 (F5) and Fragment (F6) adequately fulfilled the sampling requirement. Other two riparian forest fragments surrounding the forest of Kurmin Danko and coded as Kurmin Danko 3 (KD3) and Kurmin Danko 4 (KD4) were also used for the study. It is worth noting that the search for individuals was conducted in many other fragments but only in those listed here the species were found or the minimum required number of samples was met. In the case of *Cardioglossa schioetzi* only three males were heard in two fragments in Kurmin Danko which was considered insufficient for those sites to be included in the study. Tissue samples of the four

species (Table 4-1) were preserved in microtubes with 95% ethanol for two months and then genetic analysis was carried out at the University of Canterbury in New Zealand.

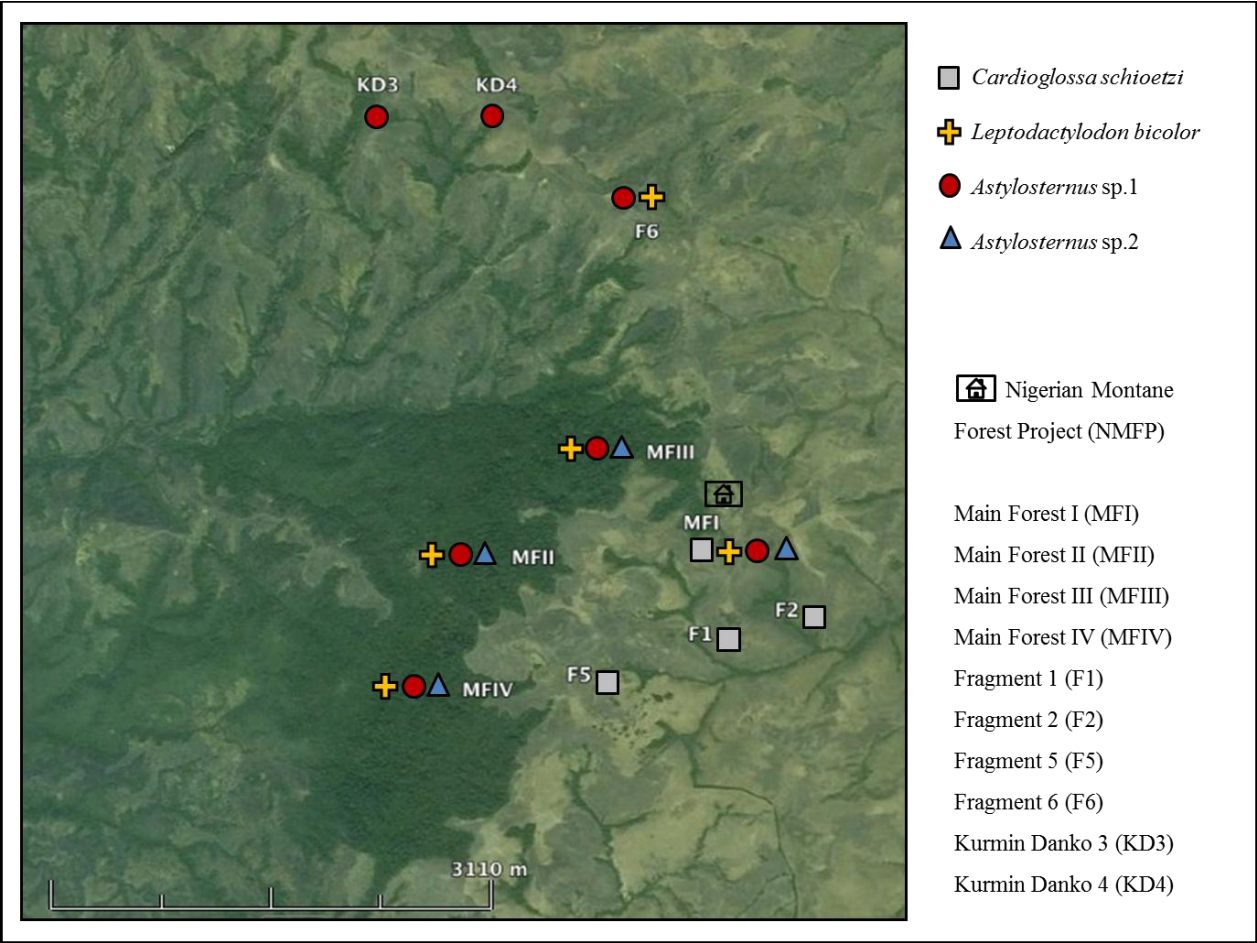


Figure 4-1. Sampling sites/Localities sampled for the frog species of interest. Geographic distribution varies among species. It is also shown the Nigerian Montane Forest Project (NMFP) field station (Google earth, 2014).



Table 4-1. Number of samples per species per site. Main Forest I (MFI), Main Forest II (MFII), Main Forest III (MFIII), Main Forest IV (MFIV), Fragment 1 (F1), Fragment 2 (F2), Fragment 5 (F5), Fragment 6 (F6), Kurmin Danko 3 (KD3), Kurmin Danko 4 (KD4).

	MFI	MFII	MFIII	MFIV	F1	F2	F5	F6	KD3	KD4
<i>Cardioglossa schioetzi</i>	13				30	20	21			
<i>Leptodactylodon bicolor</i>	31	20	24	30				26		
<i>Astylosternus</i> sp. 1	8	29	7	20				25	22	28
<i>Astylosternus</i> sp. 2	17	1	18	6						

#### 4.3.2 DNA extraction and AFLP profiling

Genomic DNA was extracted from tissue samples using a modified CTAB (Cetyltrimethylammonium bromide) protocol (Weising *et al.* 1995). The AFLP protocol was based on Vos *et al.* (1995) with minor modifications. See chapter 3 for a comprehensive explanation on the technique and methods. In brief, restriction enzymes EcoRI and MseI were used to perform double digestion of the total genomic DNA. Adapters (sequences of known DNA) were ligated to the ends of the cut fragments using T4 ligase enzyme. Two increasingly selective amplification processes were applied to reduce the complexity of fragment pattern. Such amplifications were conducted using primers that match the known adapter sequence plus additional selective nucleotides (Table 4-2). The length of the amplified fragments was detected on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Table 4-2. Restriction enzymes, adapters and primers sequences used on the AFLP procedure.

		Sequence (5'-3')
Restriction enzymes	EcoRI	G <sup>^</sup> AATTC
		CTTAA <sup>^</sup> G
	MseI	T <sup>^</sup> TAA
		AAT <sup>^</sup> T
Adapters	EA2	CTCGTAGACTGCGTACC
	EA3	AATTGGTACGCAGTCTAC
	MA1	GACGATGAGTCCTGAG
	MA2	TACTCAGGACTCAT
Pre-selective primers	ENP	GACTGCGTACCAATT
	MNP	GATGAGTCCTGAGTAA
Selective Primers	ESP1B	GACTGCGTACCAATTCAG*
	MSP3	GATGAGTCCTGAGTAACGAT
	MSP6	GATGAGTCCTGAGTAACCTC

\*Labeled with fluorescence.

Amplified fragments were visualized as peaks or electropherograms where each peak represents a fragment of a precise length. GENEMAPPER 4 (Applied Biosystems) was used to analyze the fragments, and a binary matrix of presence (1) and absence (0) was

built. Only unambiguously scorable loci were included in the analysis. Samples with missing data were not used for genetic analysis.

#### **4.4 Data analysis**

GenAlEx v6.5 (Peakall and Smouse 2012) was used to perform frequency and distance-based analyses. Allelic frequencies were estimated following the Lynch and Milligan (1994) method assuming independent nuclear loci and Hardy–Weinberg equilibrium within populations. The expected Heterozygosity ( $H_e$ ) was calculated as  $2(p)(q)$  implemented for diploid binary data (dominant markers) and assuming random mating, where  $q = (1 - \text{Band Frequency})^{0.5}$  and  $p = 1 - q$ .

The genetic data were converted into a pairwise individual-by-individual genetic distance matrix which was then used to assess the genetic structure within populations. This is a true Euclidean metric (see Peakall and Smouse 2012) as required for the subsequent analysis of molecular variance.

An Analysis of Molecular Variance (AMOVA) was carried out to investigate the hierarchical partitioning of genetic variation among populations and estimate the population genetic differentiation via  $\Phi_{PT}$ . This is a measure analogous to  $F_{ST}$  that is used for dominant markers such as AFLP (Peakall *et al.* 1995).  $\Phi_{PT}$  was calculated as  $V_{AP}/(V_{AP} + V_{WP})$ , where  $V_{AP}$  is the variance among populations and  $V_{WP}$  the variance within populations. A nonparametric permutation method (Excoffier *et al.* 1992) was used for testing statistical significance of the  $\Phi_{PT}$  values, with the number of permutations set to 999.

Principal Coordinates Analysis (PCoA) was performed to assess and visualize the patterns of genetic relationships among individuals using a genetic distance matrix

generated from the binary presence-absence matrix, and among populations based on the  $\Phi_{PT}$  pairwise matrix. PCoA was performed via covariance matrices with data standardization using GenAlEx v6.5.

The genetic structure was further investigated following the Bayesian model-based clustering algorithms implemented in Structure v2.3.3 (Pritchard *et al.* 2000). Structure uses a Markov chain Monte Carlo (MCMC) algorithm to cluster individuals into populations based on their genotypes. It generates posterior probabilities of assignment of individuals to each of a given number of  $K$  groups or populations regardless of their site of origin (Pritchard *et al.* 2000). For each species, Structure was run first using all populations across the entire study area, that is, without prior information model. Then the population data (sampling sites) were employed as prior information to assist the clustering as recommended when the signal of structure is weak (Pritchard *et al.* 2000, Hubisz *et al.* 2009). For each of the target species a different range of  $K$  values were determined by the number of sampling sites (e.g. varying  $K$  from one to four for *C. schioetzi*, but one to seven for *A. A. sp. 1*) was set. Thus, batches of 5 independent runs with a burn-in of 10,000 and 100,000 iterations and specific range of  $K$  values (depending on the species) were applied. A model of admixture with correlated allele frequencies was applied. The web version of Structure Harvester (Earl and vonHoldt 2012) was used to calculate the ‘optimal’  $K$  or the number of genetic clusters that fit the data. The two methods to estimate the best  $K$ , the log posterior probability of the data  $Ln(K)$  given  $K$  genetic clusters (Pritchard *et al.* 2000) and  $\Delta K$  (Evanno *et al.* 2005), based on the rate of change in the log probability of data between successive  $K$  values were reported.

Structure was also employed to test for migrants for each of the species. In order to identify immigrants or individuals with recent immigrant ancestry in the last two

generations, a model using population information with correlated allele frequencies was applied. The model was set to GENSBACK = 2, and as suggested by Falush *et al.* (2007), MIGRPRIOR = 0.001 to guarantee that there is strong statistical support for any inference of mixed ancestry, thus, the prior probability that an individual has pure ancestry from its predefined population is 0.999.

To examine the pattern of isolation by distance, the correlation between geographic distance and genetic distance matrices was tested using a Mantel test as implemented in GenAlEx v6.5. The geographic distance matrix (distance among standard coordinates, X matrix) was estimated by the formula

$$D = \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2}$$

where,  $x_i$  and  $y_i$  are the coordinates for the  $i$ -th sample and  $x_j$  and  $y_j$  are the coordinates for the  $j$ -th sample. The  $\Phi_{PT}$  pairwise matrix was used as the genetic distance matrix (Y matrix). In order to test the significance of such matrices a correlation coefficient for the two data matrices with a test for a significant relationship by random permutation (999) was generated.

Although the two programs GenAlEx and Structure can handle missing data, missing genotypes were removed from the analyses for the two preferred combinations of primers for all the target species for all the analyses. It has been recognized that missing data could be problematic for some pairwise distance-based analyses implemented in GenAlEx (Peakall and Smouse 2006). Even though this program provides an option to interpolate the genetic distance for missing loci an excessive bias is prevented by eliminating missing data from the analyses (see Peakall and Smouse 2012).

## 4.5 Results

The two used primer combinations ESP1B/MSP3 and ESP1B/MSP6 yielded 631 loci for the entire 396 samples, that is, the total number of samples including the four species. Out of the 631 loci, 554 (87.7%) were polymorphic. The number of polymorphic loci per species generated by the ESP1B/MSP3 combination ranged from 51 to 83, whereas for the pair ESP1B/MSP6 the polymorphic loci ranged from 67 to 108. For both markers, the minimum number of polymorphic loci was recorded for *Leptodactylodon bicolor* and the maximum for *Astylosternus* sp. 1. More details were presented in chapter 3.

In order to simplify the results the genetic characterization is presented separately for each of the four frog species.

### 4.5.1 *Cardioglossa schioetzi*

Table 4-3 summarizes the genetic diversity of *C. schioetzi*. The percentage of polymorphic loci ranged from 66.4% for MFI to 84% for F1; overall for the four populations of this frog species the percentage of polymorphic loci was 74.8% (Standard Error SE = 3.8). The population containing the minimum number of private peaks (three) was F2, whereas the maximum was seven, reported for F1. The average of genetic diversity ( $H_e$ ) over populations and loci was 0.219 (SE = 0.008). F5 had the highest genetic diversity (0.229, SE = 0.016), and F2 the lowest (0.199, SE = 0.017), however, the genetic diversity between populations was not significantly different ( $F(3) = 0.65$ ,  $P = 0.58$ ).

Table 4-3. Sample size (N), Number of Different Peaks (NDP), Number of Private Peaks (NPP), Percentage of Polymorphic Loci (PPL), and Expected Heterozygosity (He) or Genetic Diversity. The standard error (SE) is in brackets and was calculated over loci for each population.

Population	N	NDP	NPP	PPL	He
MFI	13	87	7	66.4	0.221(0.019)
F1	28	110	5	84	0.227 (0.016)
F2	20	105	3	71.20	0.199 (0.017)
F5	21	102	3	77.60	0.229 (0.016)
Mean				74.80 (3.83)	0.219 (0.008)

Populations are coded as MFI (Main Forest I), F1 (Fragment 1), F2 (Fragment 2), F5 (Fragment 5).

The AMOVA provided a general framework for the analysis of population genetic structure. Results of the partitioning of molecular variance within and among populations indicated that 98% of the variation is within the populations and only 2% among populations.

Based on the  $\Phi_{PT}$  calculated via AMOVA, no significant genetic differentiation was detected among the populations ( $\Phi_{PT} = 0.018$ ,  $p = 0.097$ ). On the other hand, pairwise estimates of  $\Phi_{PT}$  among populations showed differences between the population from the forest MFI with each of the riparian fragments F1, F2, and F5, but no significant difference among these three riparian fragments was recorded (Table 4-4).

Table 4-4. Pairwise matrix of  $\Phi_{PT}$ .  $\Phi_{PT}$  values below diagonal, and above it probability values.

	MF1	F1	F2	F5
MF1	-	0.026	0.042	0.005
F1	0.051	-	0.424	0.398
F2	0.061	0.000	-	0.371
F5	0.101	0.000	0.000	-

The outcome of the PCoA analysis based on the  $\Phi_{PT}$  pairwise matrix is not presented here as it revealed no clusters (all of the variation was explained in the first axis). The PCoA based on the individual by individual genetic distance matrix is showed in figure 4-2. The scatter plot showed two clouds of data points. Individuals from MFI were present in all except one of the quadrants (lower right quadrant), however, individuals of riparian fragment populations F1, F2 and F5 were dispersed in the four quadrants. The first and second coordinates accounted for 25.10% and 13.95% of the total variation respectively.

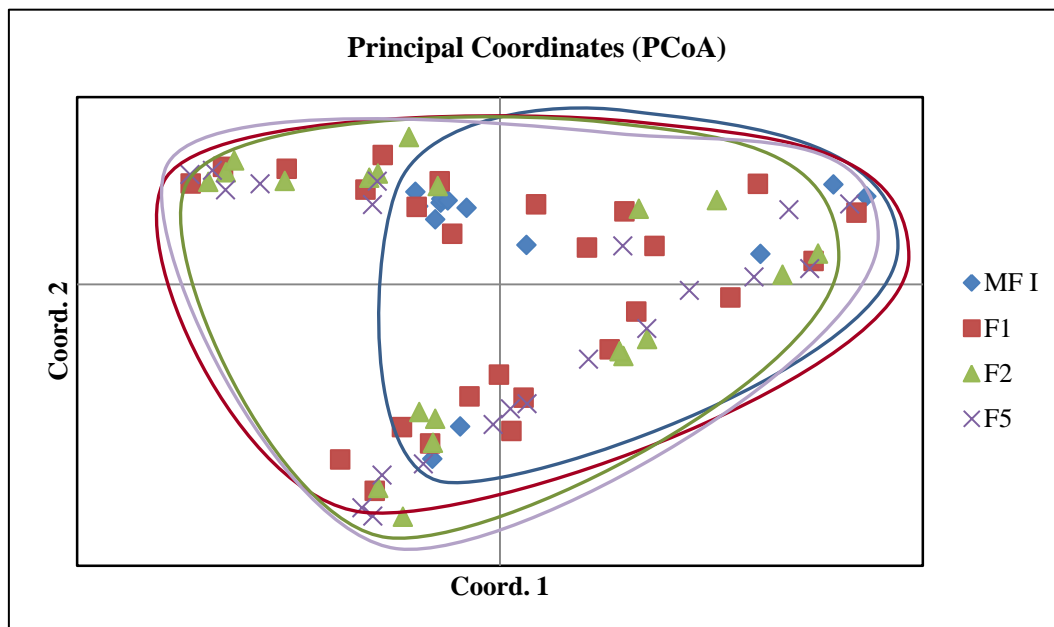


Figure 4-2. PCoA plot based on the individual distance matrix. Patterns of two axes of variation within the genetic data set of *Cardioglossa schioetzi*. Each point represents an individual. Sampling sites are color-coded.

Analysis in Structure revealed that populations have admixed ancestry because inferred clusters consisted of individuals from several distinct populations. With the first run using all the sites across the study area (Figure 4-3) the optimal  $K$  based on the  $Ln(K)$  was  $K = 4$ , and  $\Delta K = 2$  according to Evanno's method. When using prior information



(Figure 4-4),  $K = 4$  and  $\Delta K = 3$  were recognized as the best  $K$  for each of the methods respectively. Like the first run, this analysis generated groups formed with individuals from several distinct populations or sampling sites.

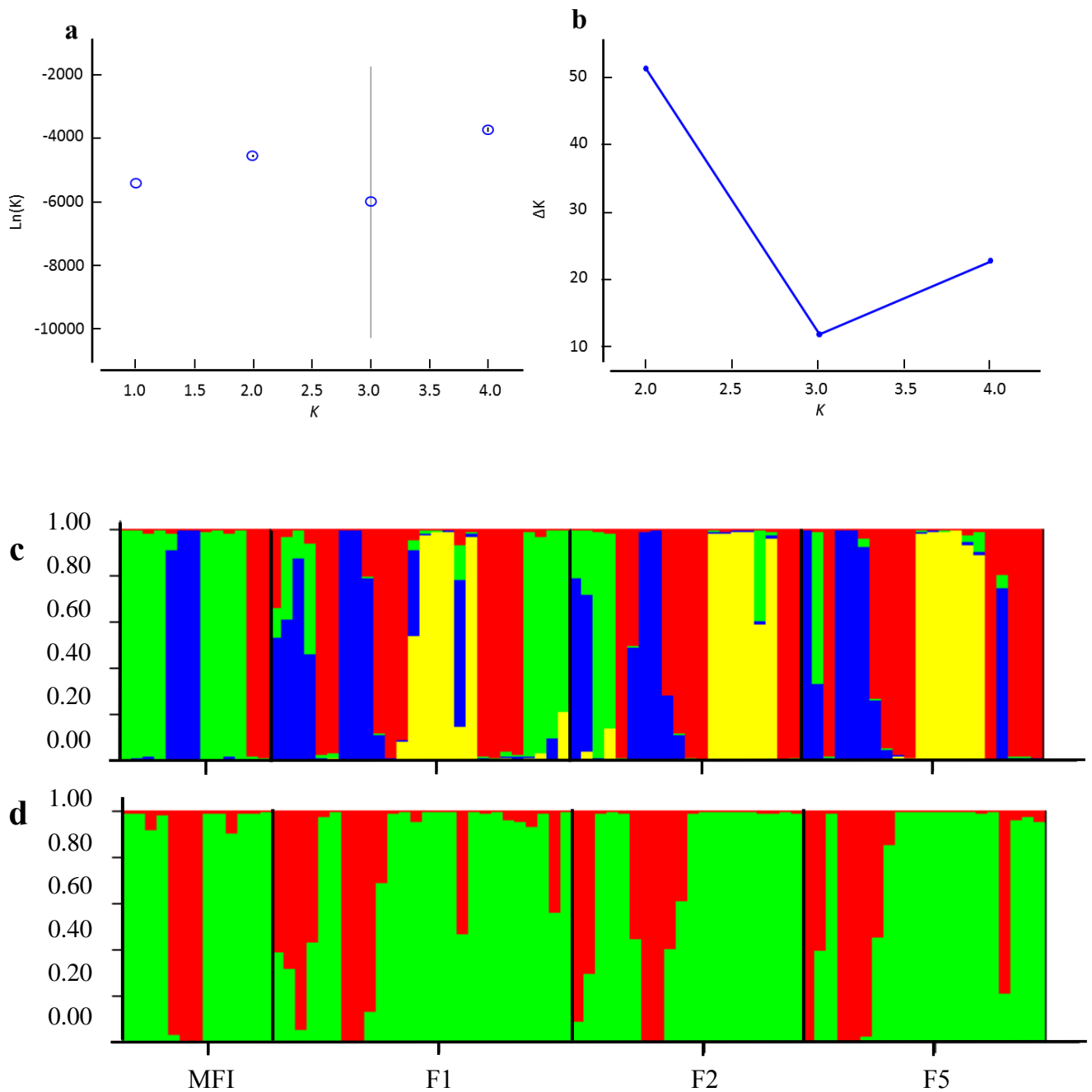


Figure 4-3. Outcomes from Structure without using population data for *Cardioglossa schioetzi*. Graph a) and bar plot c) depict the optimal  $K$  based on the  $\ln(K)$   $K = 4$ , whereas graph b) and bar plot d) show the better  $K$  based on Evanno's method  $\Delta K = 2$ . Each vertical bar represents an individual for which is shown the proportional genetic assignment to each cluster.

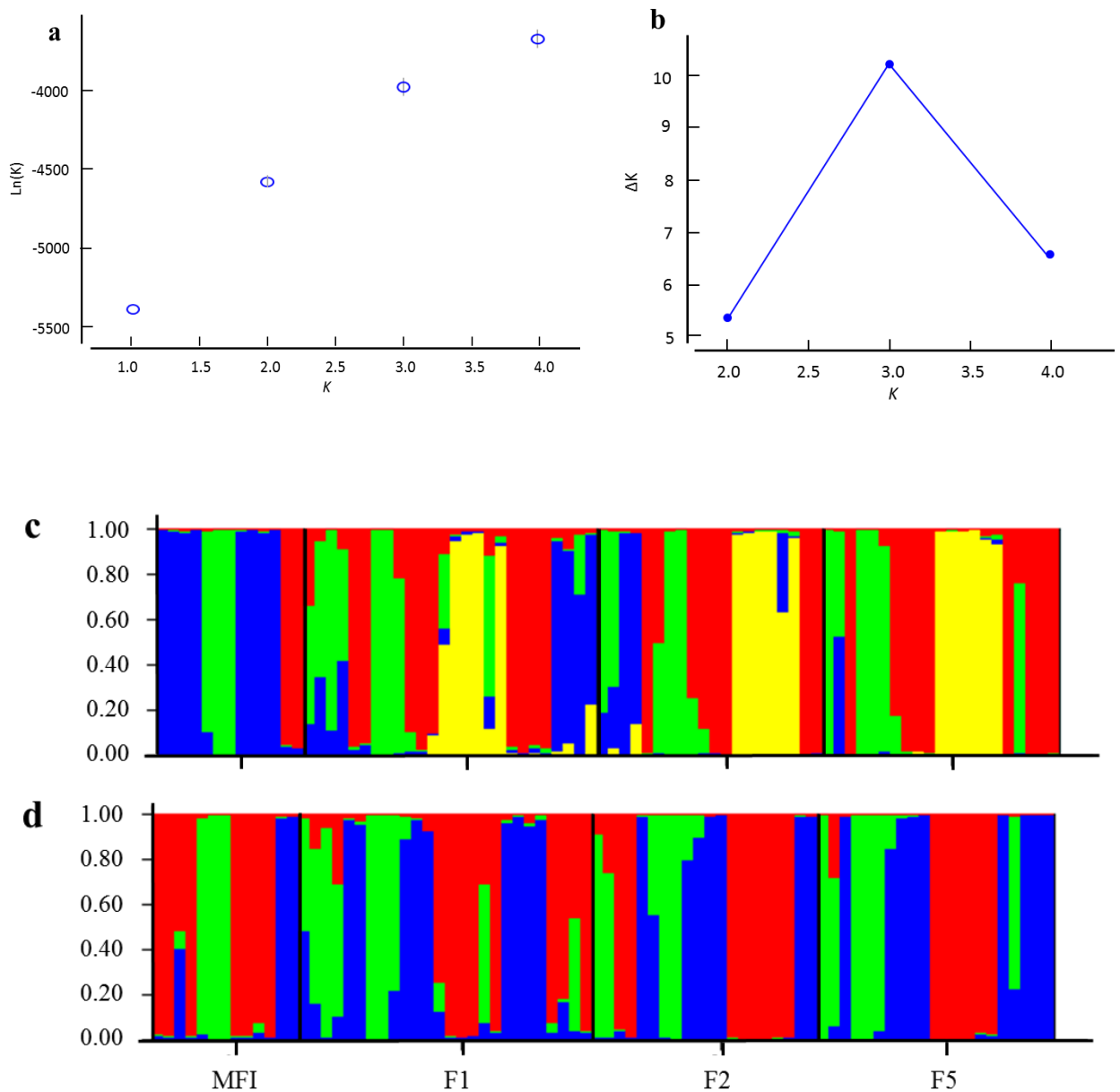


Figure 4-4. Structure assignments using sampling location information. a)  $K = 4$  clusters based on the  $\ln(K)$ , and b) following Evanno's method shows the assignment into  $\Delta K = 3$  clusters. The bar plots at the bottom show  $K = 4$  and d)  $\Delta K = 3$ . Each vertical bar represents an individual for which is shown the proportional genetic assignment.

The migration test performed in Structure revealed that from the 80 individuals of the four sampled populations of *Cardioglossa schioetzi*, only 27 (33.75%) were actually from their presumed populations, and 53 (66.25%) were immigrants or had ancestry in

other populations in the last two generations. As for the forest population MFI only three (23%) individuals were correctly assigned, eight (61.6%) were immigrants from F1, and two (15.4%) were immigrants from F5. With regard to the riparian fragments, the population F1 with 26 individuals in total, 10 (38.46%) were from their presumed populations, whereas 16 (61.54%) were immigrants from or had ancestry in the forest population MFI (three individuals or 11.53%), and from the other two riparian fragments F2 (six individuals or 23%), and F5 (seven individuals or 27%). Riparian fragment population F2 consisted of 20 individuals in total, six (30%) of which were correctly assigned, three (15%) were immigrants from or had ancestry in MFI, six (30%) from F1 and five (25%) from F5. Finally, the population F5 had five (23.80%) immigrants or ancestry in MFI, two (9.5%) immigrants from or ancestry in F1, and six (28.6%) immigrants or ancestry in F2, while the remaining 8 (38.1%) individuals were from their presumed populations. Therefore, from the 53 immigrants (or having ancestry in other population) 21 (39.62%) were between forest and riparian fragments, while 32 (60.38%) were between the riparian fragments. Migration results between forest populations could not be obtained as *C. schioetzi* was found only in the forest population MFI.

With regard to the isolation by distance analysis, a positive relationship between the geographic distance and genetic distance matrices (using  $\Phi_{PT}$ ) was observed, however, the relationship was not significant ( $r = 0.226$ ,  $P = 0.432$ ) (Figure 4-5).

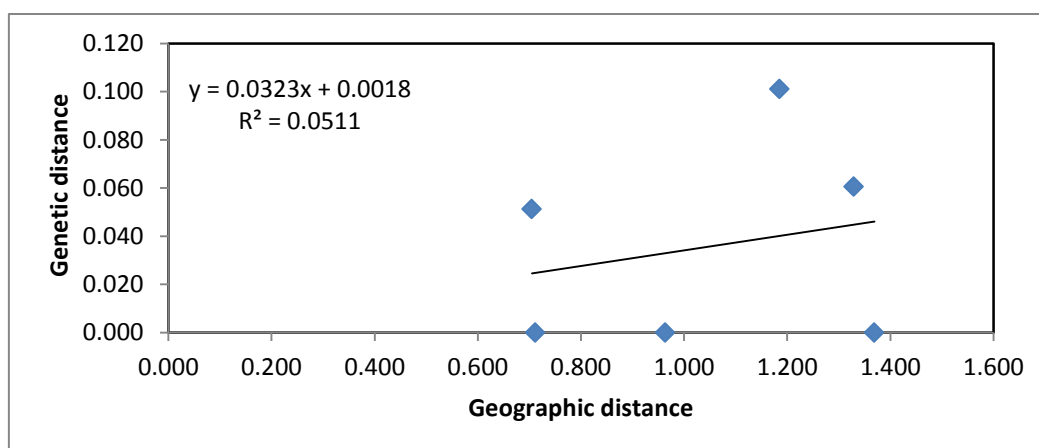


Figure 4-5. Isolation by distance for *Cardioglossa schioetzi*. The geographic distance was plotted against the genetic distance ( $\Phi_{PT}$ ) and no significant correlation was detected.

#### 4.5.2 *Leptodactylodon bicolor*

*Leptodactylodon bicolor* was abundant within the forest and was recorded in only one riparian forest fragment. Similar diversity values were obtained for the five populations (Table 4-5). The highest percentage of polymorphic loci was 76.27 for MFI, whereas the lowest was 65.25 for MFII. The highest genetic diversity level ( $H_e = 0.238$ ,  $SE = 0.018$ ) was obtained for MFIII and the lowest ( $H_e = 0.184$ ,  $SE = 0.017$ ) for F6, the riparian forest fragment population. Average heterozygosity was not significantly different among the five populations ( $F(4) = 1.28$ ,  $P = 0.28$ ).

The AMOVA revealed that 97% of the variation was within the populations and only 3% among populations. The  $\Phi_{PT}$  estimated was 0.026 and significant ( $P = 0.020$ ) genetic differentiation was detected among the five populations. The  $\Phi_{PT}$  pairwise matrix (Table 4-6) showed significant genetic differences between F6 and MFI and MFII.

Table 4-5. Sample size (N), Number of Different Peaks (NDP), Number of Private Peaks (NPP), Percentage of Polymorphic Loci (PPL), and Expected Heterozygosity (He) or Genetic Diversity. The standard error (SE) is in brackets and was calculated over loci for each population.

<b>Population</b>	<b>N</b>	<b>NDP</b>	<b>NPP</b>	<b>PPL</b>	<b>He</b>
MFI	31	97	5	76.27	0.217(0.017)
MFII	20	98	6	65.25	0.198 (0.018)
MFIII	19	94	2	74.58	0.238 (0.018)
MFIV	25	90	3	66.10	0.208 (0.018)
F6	23	92	3	66.95	0.184 (0.017)
Mean				69.83 (2.31)	0.209 (0.008)

Table 4-6. Pairwise matrix of  $\Phi_{PT}$ .  $\Phi_{PT}$  values below diagonal, and above it probability values.

	<b>MFI</b>	<b>MFII</b>	<b>MFIII</b>	<b>MFIV</b>	<b>F6</b>
<b>MFI</b>	-	0.103	0.260	0.280	0.013
<b>MFII</b>	0.021	-	0.112	0.366	0.007
<b>MFIII</b>	0.007	0.029	-	0.224	0.103
<b>MFIV</b>	0.003	0.001	0.011	-	0.055
<b>F6</b>	0.054	0.073	0.027	0.039	-

The  $\Phi_{PT}$  matrix, visualized through a PCoA (Figure 4-6), shows the genetic difference between the riparian fragment population F6 and two of the main forest populations, MFI and MFII.

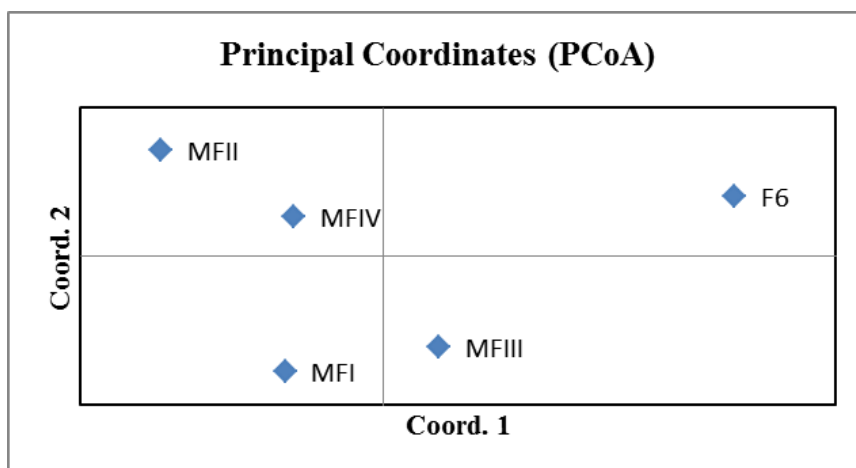


Figure 4-6. PCoA plot of the first two axes based on the  $\Phi_{PT}$  pairwise matrix. Patterns of genetic relationships among populations of *Leptodactylodon bicolor* based on the  $\Phi_{PT}$  pairwise matrix.

The PCoA based on the individual genetic distance matrix (Figure 4-7) revealed the dispersion of the individuals based on the two first axes. Individuals were spread between the four quadrants and at least four discrete clusters were observed. These clusters shown on Figure 4-7 are not related to sampling sites. The first two axes explained 41.33% (27.51% and 13.82% respectively) of the total variation.

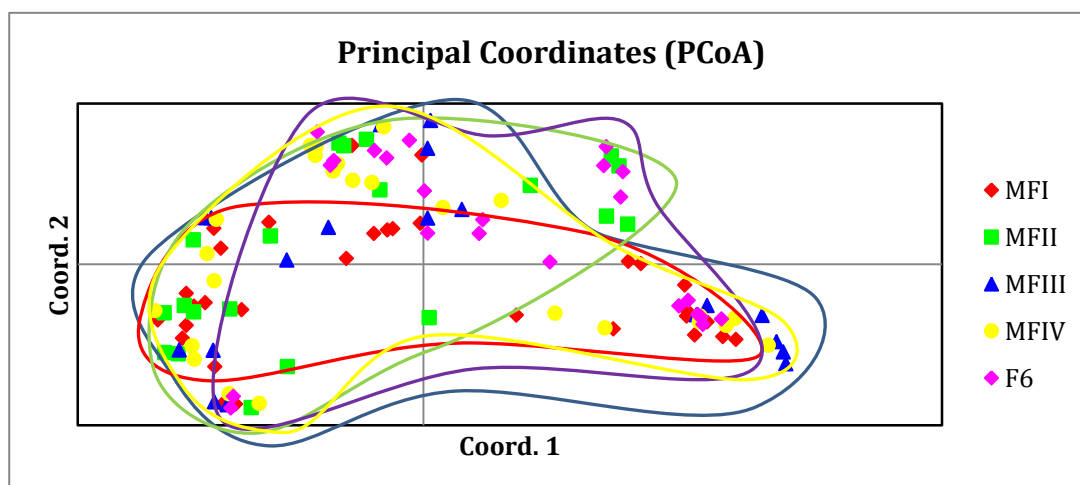


Figure 4-7. PCoA plot showing the first two axes based on the 118 genetic profiles of *Leptodactylodon bicolor*. Sampling sites are encircled by color code.

The assignment results from the two runs in Structure (employing all sites and prior information, respectively) yielded the same information. Based on the  $\ln(K)$  estimation five genetic groups ( $K = 5$ ) (Figure 4-8a and 4-8c) were detected, whereas the Evanno's estimation detected two clusters ( $\Delta K = 2$ ) (Figure 4-8b and 4-8d).

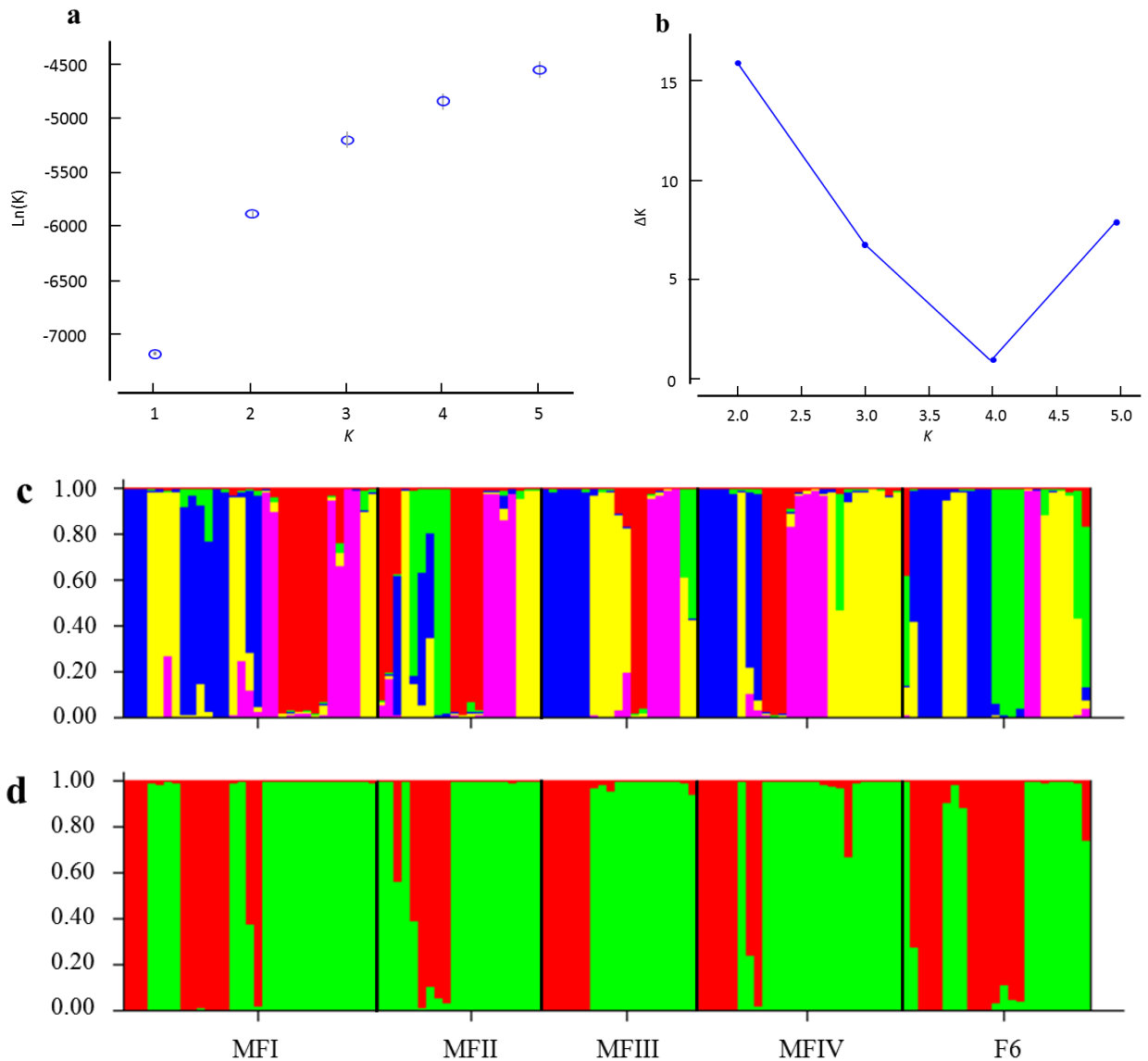


Figure 4-8. Structure estimates of  $K$  groups for *Leptodactylodon bicolor*. Graph a) and bar plot c) show  $K = 5$  based on the  $\ln(K)$  estimation. b and d) represent respectively the graph and plot of the best  $K$  following Evanno's approach  $\Delta K = 2$ . The two Structure runs (with and without prior information) yielded the same information. Each vertical bar represents an individual for which is shown the proportional genetic assignment.

The test for migrants in Structure indicated that 40 individuals (33.90%) of *Leptodactylodon bicolor* were from their presumed populations, while 78 (66.10%) of the individuals were immigrants or had ancestry in other populations in the past two generations.

From these 78 immigrants 21 (26.93%) were between forest and the riparian fragment population, and 57 (73.07%) were between the forest populations. As *L. bicolor* was recorded only from F6 the migration results between riparian fragments are not reported here. The forest population MFI had immigrants from the other forest populations, specifically six individuals (19.35%) from MFII, 10 (32.25%) from MFIII, eight (25.80%) from MFIV, and from the riparian fragment F6 one (3.22%) individual. For the population MFII, six (30%) individuals were correctly assigned, whereas four (20%) immigrants were from MFI, four (20%) from MFIV, and six (30%) were from F6. For the population MFIII with 19 individuals in total, 13 (68.42%) were immigrants from MFI (4 individuals, 21.05%), MFII (2 individuals, 10.52%), MFIV (5 individuals, 26.31%) and F6 (2 individuals, 10.53%), and six (31.58%) were from the presumed population. As for the population MFIV, 10 (40%) individuals were correctly assigned, five (20%) were immigrants from MFI, three (12%) from MFII, six (24%) from MFIII, and from F6 only one (4%) individual. For the riparian fragment F6 52.17% (12 individuals) were from the presumed population, and immigrants from all the forest populations except MFII were detected. Specifically, two (8.69%) individuals were immigrants from MFI, six (26.08%) from MFIII, and three (13.04%) from MFIV.

No significant patterns of isolation-by-distance were detected. The Mantel test revealed a positive but not significant correlation between the genetic and geographical distance data sets among the populations ( $r = 0.511$ ,  $P = 0.134$ ) (Figure 4-9).



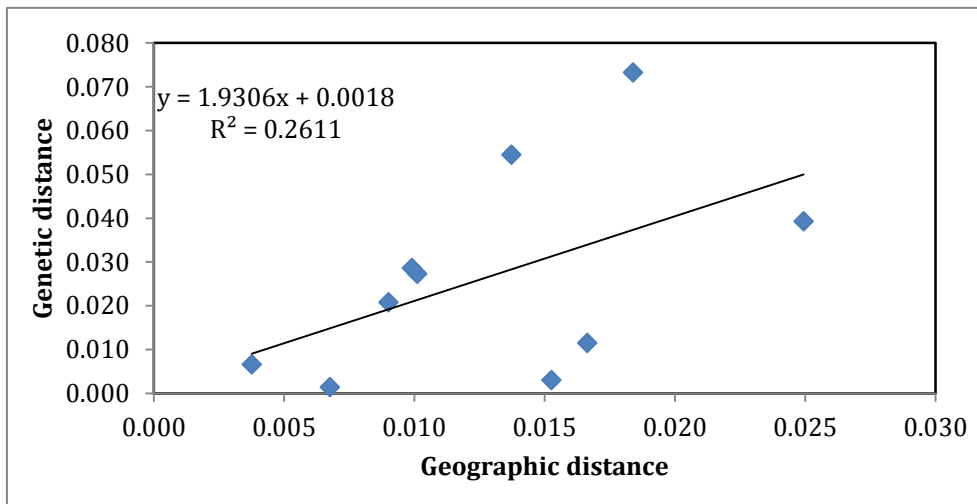


Figure 4-9. Scatter plot of  $\Phi_{PT}$  pairwise matrix and the geographic distances for all the five populations of *L. bicolor*.

#### 4.5.3 *Astylosternus sp. 1*

This frog species was abundant and among the four target species it was the most widely distributed within the study area. Seven sites were sampled, but unfortunately for the sampling sites/populations MFI and MFIII (within the forest in Ngel Nyaki) less than 10 AFLP profiles were obtained for the genetic analysis. These populations contained the two lowest percentages of polymorphic loci (51.31 and 52.88% respectively). The population with the highest percentage of polymorphic loci (79.58) was KD4. The genetic diversity values ranged from 0.142 (SE = 0.012) for MFI to 0.181(SE = 0.012) for MFIV (Table 4-7).

Table 4-7. Summary of peak/band patterns and genetic diversity across populations. Sample size (N), Number of Different Peaks (NDP), Number of Private Peaks (NPP), Percentage of Polymorphic Loci (PPL), and Expected Heterozygosity (He) or Genetic Diversity. The standard error (SE) is in brackets and was calculated over loci for each population.

<b>Population</b>	<b>N</b>	<b>NDP</b>	<b>NPP</b>	<b>PPL</b>	<b>He</b>
MFI	8	101	1	51.31	0.142(0.012)
MFII	28	154	2	79.06	0.167 (0.012)
MFIII	6	105	0	52.88	0.170 (0.014)
MFIV	19	149	8	76.96	0.181 (0.012)
F6	24	130	1	67.02	0.178 (0.013)
KD3	21	144	3	73.82	0.180 (0.013)
KD4	27	155	5	79.58	0.173 (0.011)
Mean				68.66 (4.57)	0.170 (0.005)

Average heterozygosities of the seven populations were not significantly different ( $F(6) = 1.149$ ,  $P=0.33$ ).

The AMOVA revealed that 98% of the total genetic variation was distributed within populations and 2% among populations. The overall  $\Phi_{PT}$  value was significant ( $\Phi_{PT} = 0.017$ ,  $P = 0.005$ ) indicating differences among populations.

The pairwise matrix of genetic differentiation (Table 4-8) revealed that only two pairs of populations were significantly different, MFIV and F6 ( $\Phi_{PT} = 0.045$ ,  $P = 0.017$ ), and KD4 and F6 ( $\Phi_{PT} = 0.033$ ,  $P = 0.032$ ).

Table 4-8. Genetic differentiation between *Astylosternus* sp. 1 populations.  $\Phi_{PT}$  values below diagonal, probability values above diagonal.

	<b>MF1</b>	<b>MFII</b>	<b>MFIII</b>	<b>MFIV</b>	<b>F6</b>	<b>KD3</b>	<b>KD4</b>
<b>MF1</b>	-	0.092	0.238	0.048	0.176	0.226	0.389
<b>MFII</b>	0.031	-	0.425	0.109	0.031	0.121	0.126
<b>MFIII</b>	0.027	0.000	-	0.392	0.278	0.369	0.300
<b>MFIV</b>	0.046	0.015	0.000	-	0.017	0.075	0.161
<b>F6</b>	0.024	0.026	0.012	0.045	-	0.308	0.032
<b>KD3</b>	0.020	0.012	0.003	0.022	0.003	-	0.294
<b>KD4</b>	0.000	0.011	0.008	0.011	0.033	0.004	-

Visualization of the  $\Phi_{PT}$  pairwise matrix was performed via PCoA (Figure 4-10) where the divergence among the populations MFIV and F6, as well as KD4 and F6 was evident. The first two axes explained 87.44% of the total variation.

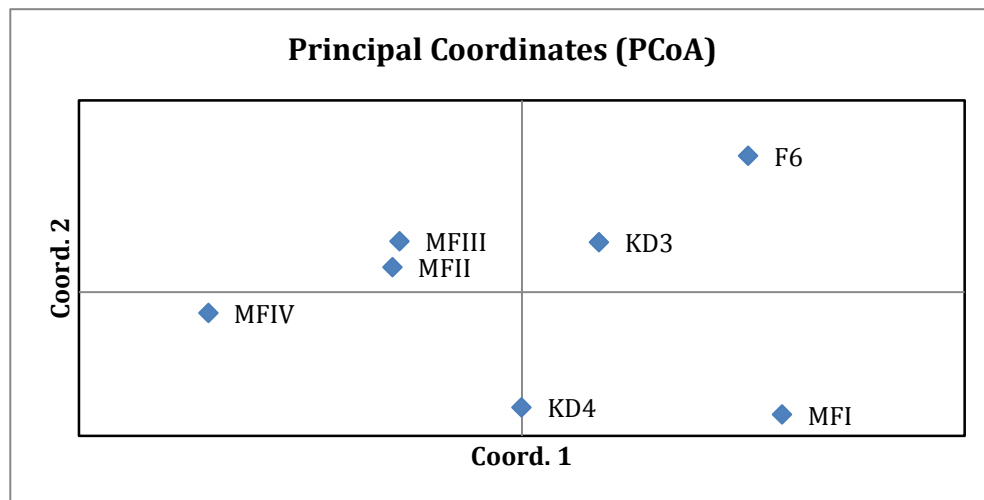


Figure 4-10. PCoA, scatter plot based on the  $\Phi_{PT}$  pairwise matrix showing the patterns of genetic relationships among populations of *Astylosternus* sp. 1.

The PCoA based on the individual by individual genetic distance matrix (Figure 4-11) revealed the major separation among individuals using the first two axes. A clear pattern

of grouping was not observed as no discrete clusters were formed. Most of the individuals were dispersed in three out of the four quadrants. Neither individuals from MFIII nor F6 were present in the right upper quadrant. Only 18.01 and 8.61% of the total variation was explained by the first and second axes respectively.

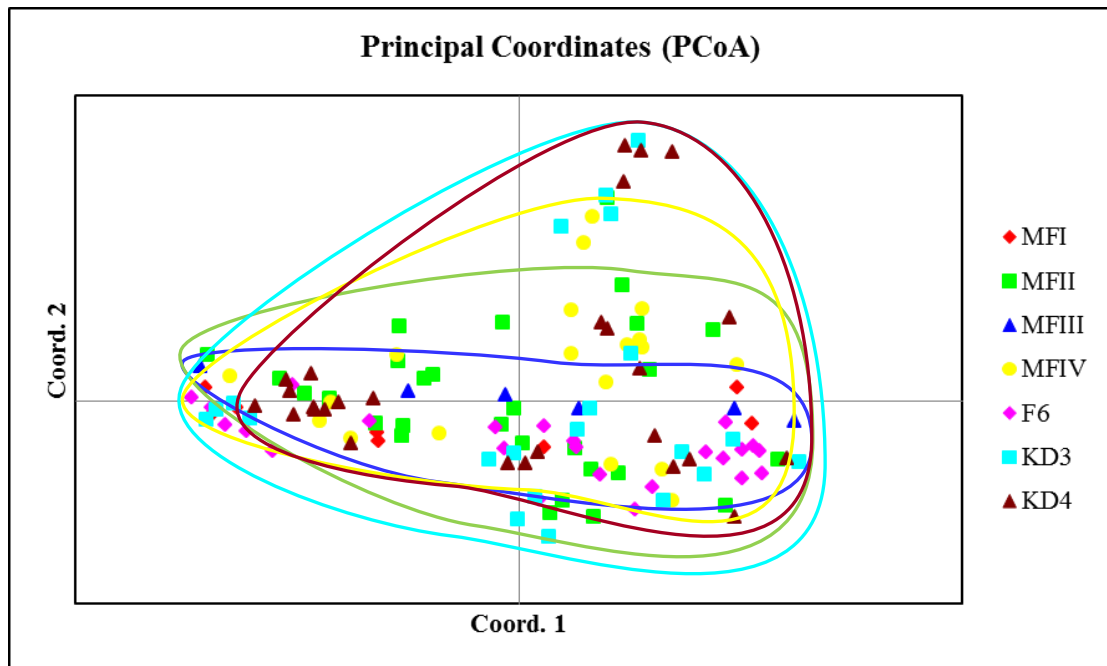


Figure 4-11. PCoA based on individual genetic distance matrix for *Astylosternus* sp. 1.

For the two runs in Structure without and using population data respectively the estimated value of  $K$  was one ( $K = 1$ ) (Figures 4-12a to 4-12d), that is, no genetic groups were detected. The estimation of  $K$  following Evanno's method detected two clusters ( $\Delta K = 2$ ) in the first run and six clusters ( $\Delta K = 6$ ) in the second one. However, these  $\Delta K$  estimates are unlikely as the method is unable to evaluate  $K$  when the highest  $\text{Ln}(K)$  occurs at  $K = 1$  (Evanno *et al.* 2005). Thus, bar plots are not presented here.

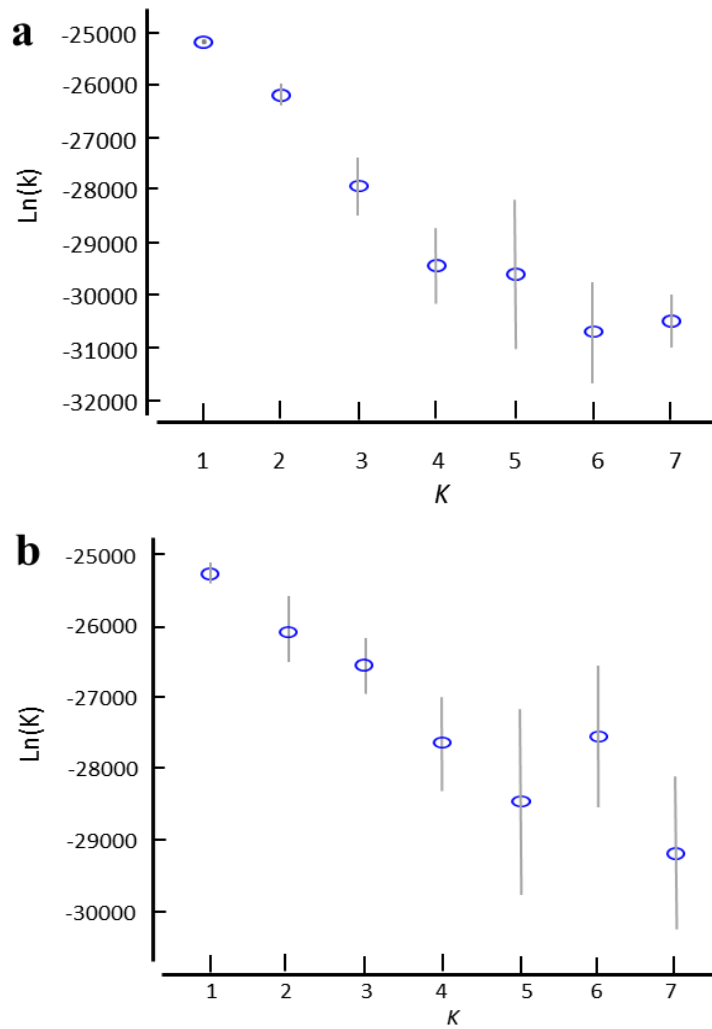


Figure 4-12. Structure assignment results for *Astylosternus* sp. 1. Graphs a) and b) show the best  $K = 1$  based on the  $\text{Ln}(K)$  for the run without prior information and using population data information respectively. Estimates of  $\Delta K$  based on Evanno's method are not shown.

A test of migration was not conducted for *Astylosternus* sp. 1 as USEPOPINFO model (using prior population information to test for migrants) in Structure does not work for ploidy  $> 2$ .

The Mantel test for isolation by distance showed no significant correlation between the genetic and geographic distance matrices ( $r = -0.121$ ,  $P = 0.297$ ) (Figure 4-13).

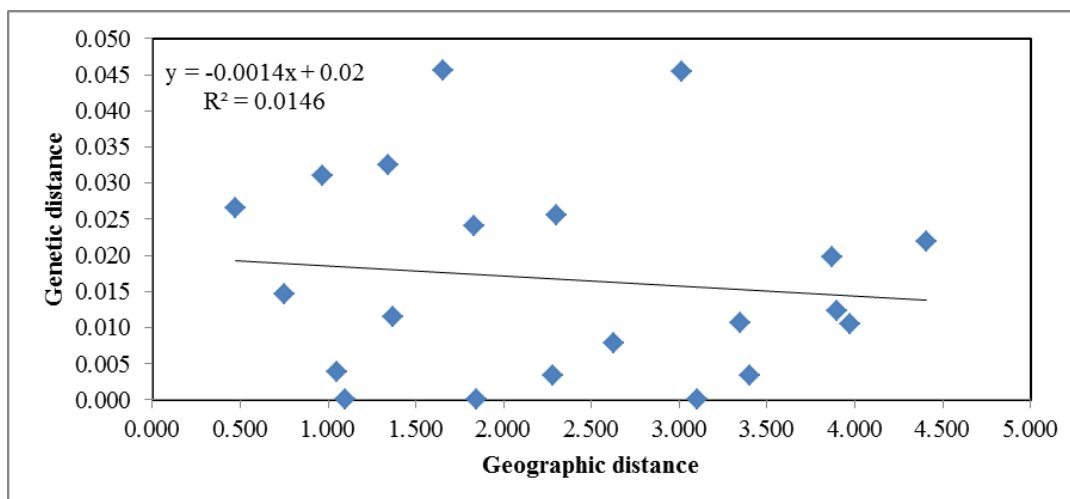


Figure 4-13. Isolation by distance. The scatter plot shows a negative and no significant correlation between pairwise  $\Phi_{PT}$  and geographic distance among populations of *Astylosternus* sp. 1.

#### 4.5.4 *Astylosternus* sp. 2

Individuals of this species were found only within the main forest in Ngel Nyaki in the three sampling sites MFI, MFIII and MFIV; unfortunately less than 20 samples were collected per site. MFII was not utilized in the analysis as only one tissue sample was obtained. Regardless of the reduced numbers of AFLP profiles used for the genetic analysis, considerable levels of genetic diversity (Table 4-9) were observed for these populations. The highest value of heterozygosity was 0.239 (SE = 0.018) for MFI and the lowest 0.168 (SE = 0.016) was observed for the population MFIV. With regard to polymorphic loci, the percentages ranged from 57.50 to 89.17 and the mean percentage was 73.33. Population MFIII contained 20 private peaks. Comparison of average heterozygosities indicated a significant difference among the three populations ( $F(2) = 5.27$ ,  $P < 0.05$ ).

Table 4-9. Summary of genetic diversity for the three populations of *Astylosternus* sp. 2. Sample size (N), Number of Different Peaks (NDP), Number of Private Peaks (NPP), Percentage of Polymorphic Loci (PPL), and Expected Heterozygosity (He) or Genetic Diversity. The standard error (SE) is in brackets and was calculated over loci for each population.

<b>Population</b>	<b>N</b>	<b>NDP</b>	<b>NPP</b>	<b>PPL</b>	<b>He</b>
MFI	16	89	3	73.33	0.239(0.018)
MFIII	18	113	20	89.17	0.229 (0.016)
MFIV	6	80	4	57.50	0.168 (0.016)
Mean				73.33 (9.14)	0.212 (0.010)

The results of the partitioning of genetic variation showed that 97% of the variation was explained within populations and 3% among populations. Like the  $\Phi_{PT}$  pairwise matrix (Table 4-10) the  $\Phi_{PT}$  value from the AMOVA revealed no significant differences among populations ( $\Phi_{PT} = 0.033$ ,  $P = 0.138$ ).

Table 4-10. Genetic differentiation among populations of *Astylosternus* sp. 2.  $\Phi_{PT}$  pairwise values and probability values are below and above of the diagonal respectively.

	<b>MFI</b>	<b>MFIII</b>	<b>MFIV</b>
<b>MFI</b>	-	0.207	0.064
<b>MFIII</b>	0.011	-	0.105
<b>MFIV</b>	0.085	0.043	-

The PCoA based on the  $\Phi_{PT}$  matrix was not included in the results as with only three populations no groups were observed. The spatial representation of the genetic distances among individuals was visualized through the PCoA in which there was not a clear pattern as shown in Figure 4-14. Individuals of the population MFI were not present in

one of the quadrants (upper right quadrant). The first and second principal coordinates accounted for 25.10% and 6.87% of total variation, respectively.

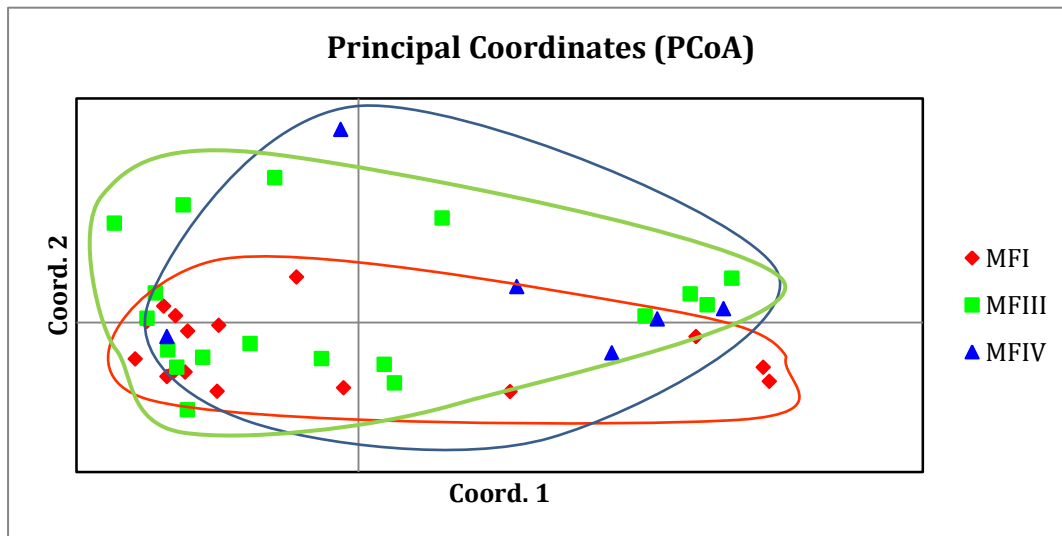


Figure 4-14. PCoA scatter plot based on the individual genetic distance matrix. Individuals are circled by color coded according to their sampling site.

The two assignment runs in Structure yielded the same results. No clusters were detected following the  $\ln(K)$  estimation ( $K = 1$ ) (Figure 4-15). Evanno's  $\Delta K$  are not shown as the most likely value cannot be evaluated if the most likely  $K$  based on the  $\ln(K)$  occurs at  $K = 1$ .



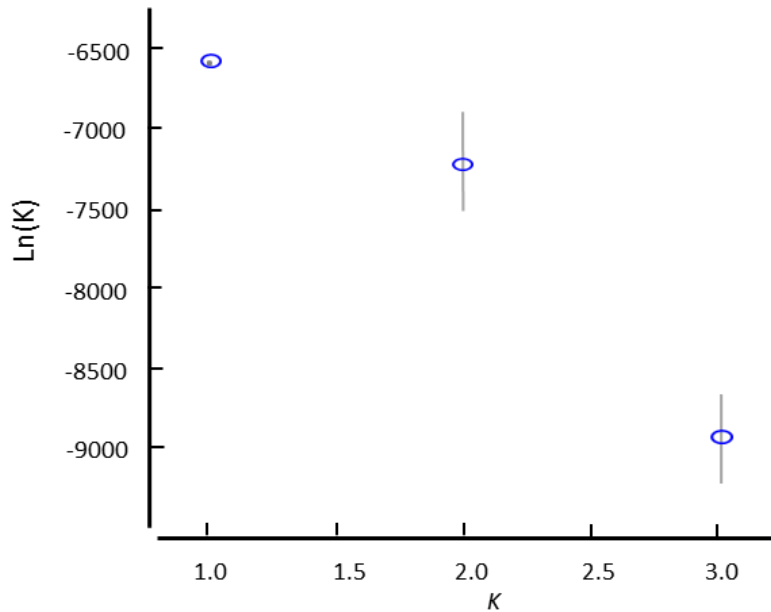


Figure 4-15. Structure analysis of *Astylosternus* sp. 2. The same results were produced with the two runs (without prior population information and using prior information respectively). The graph shows the optimal K based on the Ln (K) (K = 1), graphs and bar plots of K clusters based on Evanno's method are not presented.

Like *Astylosternus* sp. 1, the test to detect immigrants in *A.* sp. 2 was not carried out as the model USEPOPINFO to test for migrants in Structure does not work when ploidy > 2.

The isolation by distance was not calculated as the number of populations for this species was too low (only three) to produce a significant analysis.

#### 4.5.5 Comparing the genetic characterization of the four target species

Taking into account all the populations of the four target species, the highest percentage of polymorphic loci was observed for *Astylosternus* sp. 2 in MFIII (89.17%), and the lowest was 51.31% for *A.* sp. 1 in MFI. Thus, the highest and lowest percentages were observed within the main forest populations of *Astylosternus* species.

With regard to the heterozygosity, the highest and lowest values were also observed in *Astylosternus* species. Overall,  $H_e = 0.239$  ( $SE = 0.018$ ) was the highest value and it was recorded in *A. sp. 2*, while *A. sp. 1* presented the lowest value ( $H_e = 0.142$ ,  $SE = 0.012$ ), and both values were observed in the forest population MFI. The average heterozygosities ranged from 0.219 ( $SE = 0.008$ ) for *Cardioglossa schioetzi* to 0.170 ( $SE = 0.005$ ) observed in *A. sp1*. Significant differences were observed between the average heterozygosities of the four species ( $F(3) = 6.691$ ,  $P = 0.004$ ).

Comparing mean values of genetic diversity among all the species (Table 4-11), *Cardioglossa schioetzi* presented the highest percentage polymorphic loci (74.80%,  $SE = 3.83$ ) and highest value of heterozygosity (0.219,  $SE = 0.008$ ), whereas the lowest percentage (68.66%,  $SE = 4.57$ ) and heterozygosity (0.170,  $SE = 0.005$ ) were observed in *Astylosternus sp. 1*.

Table 4-11. Sampled populations (SP), Number of individuals analyzed (NA), average Percentage of Polymorphic Loci (PPL), and average expected Heterozygosity ( $H_e$ ) for each species. In brackets is the standard error (SE).

Species	SP	NA	PPL	$H_e$
<i>Cardioglossa schioetzi</i>	4	80	74.80 (3.83)	0.219 (0.008)
<i>Leptodactylodon bicolor</i>	5	118	69.83 (2.31)	0.209 (0.008)
<i>Astylosternus sp. 1</i>	7	133	68.66 (4.57)	0.170 (0.005)
<i>Astylosternus sp. 2</i>	3	40	73.33 (9.14)	0.212 (0.010)

The population genetic differentiation of the four frog species is summarized in Table 4-12.

The AMOVA revealed significant genetic differentiation among populations of *Leptodactylodon bicolor* and *Astylosternus* sp. 1. The  $\Phi_{PT}$  pairwise indicated significant differences among forest and riparian fragment populations for *Cardioglossa schioetzi*, *L. bicolor* and *A. sp. 1*. No genetic differences were detected among populations within the forest for any of the species.

Table 4-12. Population genetic differentiation for each species Overall  $\Phi_{PT}$  and probability values, plus range of pairwise  $\Phi_{PT}$  values.

Species	Overall $\Phi_{PT}$	p	$\Phi_{PT}$ ranges
<i>Cardioglossa schioetzi</i>	0.018	0.097	0.051 – 0.101
<i>Leptodactylodon bicolor</i>	0.026	0.020	0.001 – 0.073
<i>Astylosternus</i> sp. 1	0.017	0.005	0.003 – 0.046
<i>Astylosernus</i> sp. 2	0.033	0.138	0.011 – 0.085

The structure test for migration (Table 4-13) revealed that for *Cardioglossa schioetzi* 53 out of the 80 individuals were immigrants or had ancestry in other populations in the past two generations. Specifically, migration between forest and riparian fragments was 39.62% (21 individuals), and between the riparian fragments was 60.38% (32 individuals). As for *Leptodactylodon bicolor*, 78 (66.10%) of the individuals were immigrants or had ancestry in other populations in the past two generations. Twenty one (26.93%) of which were between forest and the riparian fragment population, whereas 57 (73.07%) were between the forest populations.

Table 4-13. Summary of the migration tests for *Cardioglossa schioetzi* and *Leptodactylodon bicolor*. Total number of individuals (N), Immigrants (I), individuals and percentage of migration between forest and riparian fragments (F↔Rf), between forest (F↔F), and between riparian fragments (Rf↔Rf).

	N	I	(F↔Rf)	(F↔F)	(Rf↔Rf)
<i>Cardioglossa schioetzi</i>	80	53	21 (39.62%)	-	32 (60.38%)
<i>Leptodactylodon bicolor</i>	118	78	21 (26.93%)	57 (73.07%)	-

#### 4.6 Discussion

This study had the main objective to provide insights on the population genetic structure of four frog species within the African anuran family Arthroleptidae at a fine geographic scale in Ngel Nyaki and Kurmin Danko Forest Reserve. Findings from this study provided the genetic characterization of the frog species *Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *Astylosternus* sp. 1 and *Astylosternus* sp. 2 by employing AFLP markers. No previous studies have been reported on this matter, thus the present study represents the first attempt made to describe genetic diversity and population genetic structure of these four species.

A very useful feature of AFLP was that fingerprints were simultaneously generated for diploid and polyploid species (*Astylosternus* sp. 1 and *A.* sp. 2). Moreover, AFLP revealed a high number of polymorphic DNA fragments. The two preferred primer combinations ESP1B/MSP3 and ESP1B/MSP6 yielded 631 loci, and 554 of these (87.7%) were polymorphic. Even though different fragments in the different species were compared, results revealed that the three species *Cardioglossa schioetzi*, *Leptodactylodon bicolor*, *A.* sp. 1 showed differentiation among the forest and the

riparian fragment populations. On the other hand, no significant genetic differentiation was detected among the forest populations for any of the four frog species.

#### **4.6.1 Genetic diversity**

Surprisingly, *Cardioglossa schioetzi* having a restricted distribution within Ngel Nyaki, and proposed to be one of the species most affected by habitat degradation and fragmentation had one of the highest values of genetic diversity for all the focus species. While the expectation was to obtain evidence of impact because of habitat fragmentation, two of its riparian forest populations had respectively the second highest value of heterozygosity ( $H_e = 0.229$ ,  $SE = 0.016$ ), and the second highest value of percentage of polymorphic loci for all the populations of all the focus species.

Conversely, *Astylosternus* sp. 1 with the widest distribution in and around Ngel Nyaki and Kurmin Danko and hypothesized as the less affected by fragmentation had the lowest values of genetic diversity ( $H_e = 0.170$ ,  $SE = 0.005$ ).

Other unexpected results were obtained for *Astylosternus* sp. 2, which with only three sampled populations and small sample sizes (40 samples available for the study) had the highest values of heterozygosity and percentage of polymorphic loci ( $H_e = 0.239$ , and 89.17%, respectively) for all the populations of the four focus species. However, for the case of the *Astylosternus* species caution should be taken when interpreting the results as these species have been reported as polyploids (see below).

The levels of genetic diversity observed for the four species in this study were similar or even higher than others reported in similar studies using AFLP in amphibians. Curtis and Taylor (2003) evaluated the impact of forest clearcuts on the population structure of *Dicamptodon tenebrosus* in southwestern British Columbia, where ranges of heterozygosity ( $H_e$ ) from 0.192 to 0.380 were reported. Specifically, the results

obtained here were more similar to those recorded in recently clearcut sites (from 0.192 to 0.285). For example, for *Cardioglossa schioetzi* the  $H_e$  values ranged from 0.199 to 0.229, for *Leptodactylodon bicolor* from 0.184 to 0.238, whereas for *A. sp. 2* the  $H_e$  values ranged from 0.168 to 0.239. Nonetheless, for *Astylosternus sp. 1* lower  $H_e$  estimates were obtained here (0.142 to 0.180).

Furthermore, Mila *et al.* (2010) examined the population structure in the Pyrenean brook newt (*Calotriton asper*), and obtained low levels of genetic diversity. For the 17 different localities the average heterozygosity was 0.046, and ranges from 0.006 to 0.105 were observed. They also reported the number of polymorphic loci per locality from 44% to 61%, which was lower than the percentage reported here. For all of the populations of the four species in this study the values of polymorphic loci ranged from 51.31% to 89.17%.

The relatively low percentage of polymorphic loci observed for *Cardioglossa schioetzi* in MFI (66.4%) and *Astylosternus sp. 2* in MFI and MFIII (51.31% and 52.88% respectively) could be a result of genetic drift due to the small population size. With regard to *C. schioetzi* it should be noted that regardless of the intensive searches for individuals during the sampling stage, low catches of animals particularly in MFI (N = 13) were obtained. If a population is small in size, the allelic frequencies can experience, among different generations, unpredictable patterns of fluctuations leading to either fixation or loss of alleles (Hedrick 2005).

#### **4.6.2 Population genetic structure**

As expected, the populations from the riparian forest fragments were generally different from the forest populations. Based on the population pairwise  $\Phi_{PT}$  values at least one

pair of forest/riparian fragment was different for each of the three species having populations in both habitats. For *Cardioglossa schioetzi* the population MFI was genetically different from riparian fragments F1, F2 and F5. Likewise, the populations MFI and MFII in the forest were different from F6 for *Leptodactylodon bicolor*, and the forest population MFIV was different from the riparian fragments F6 for *Astylosternus* sp. 1.

In continuous habitats, genetic differentiation might be caused by poor dispersal ability of species and the distance between individuals (Wright 1943, Slatkin 1987). For the salamander *Plethodon cinereus* with direct-development (i.e. no aquatic larval phase) it has been demonstrated that genetic differentiation at fine scale occurs in a continuous habitat among demes or groups separated by distances as small as 200 meters (Cabe *et al.* 2007). For the study presented here the longest distance among the forest sites was 1.850 kilometers for the pair sites MFIII and MFIV, whereas the shortest was 600 meters for the pair sites MFI and MFIII. Nonetheless, no significant genetic differences were detected among the forest populations in Ngel Nyaki for any of the species. For amphibians with an aquatic larval phase it has been suggested that the movement of larvae among adjacent streams likely contributes to gene flow among populations (Lowe 2003, Robertson *et al.* 2008). Given that data on dispersal distances do not exist or have not been published for any of the target species, comparisons with other sites within their distribution area, for example montane forests of Cameroon, were not addressed.

Similar results were observed by Dixo *et al.* (2009) who compared genetic diversity of the toad *Rhinella ornata* among small and medium forest fragments that were either isolated or connected to large forest areas by corridors. A weak but significant  $F_{ST}$  was

observed between small fragments and continuous forest, however, no significant genetic differentiation was observed among continuous forest sites.

The clustering analysis in Structure indicated that in general for the four species, groupings were not consistent with sampling location, and that many of their populations and individuals were admixed. This is also demonstrated by the migration results which confirmed gene flow among populations of *Cardioglossa schioetzi* and *Leptodactylodon bicolor*.

For all the four species the structure analysis was not congruent between the  $\ln(K)$  and  $\Delta K$  estimators. In simulations  $\Delta K$  has been shown to be better at identifying the true number of clusters than  $\max \ln(K)$  ((Evanno *et al.* 2005). Results presented here on the  $\Delta K$  clusters based on Evanno's method were consistent with the PCoA analysis for *Cardioglossa schioetzi*, and the two *Astylosternus* spp., however, for *Leptodactylodon bicolor* consistency between PCoA and  $K$  based on the  $\ln(K)$  was observed. Yet, the main interest of the study was not to estimate a definitive number of genetic clusters but to compare the patterns of genetic groups between the four frog species.

According to the  $\ln(K)$ , the optimal  $K$  for the two *Astylosternus* species revealed no genetic clustering,. Although this may be an effect of the scale of sampling, the number of loci used, or both (Pritchard *et al.* 2000), it is worth noting that polyploidy could be a strong influence on these results. According to Pritchard *et al.* (2010) when estimating  $K$  when RECESSIVEALLELES mode (option for data such as AFLP and for polyploids where genotypes may be ambiguous) is used for diploids, the likelihood at each step of the Markov chain is computed by adding over the possible genotypes; however, when polyploidy  $> 2$  is used the current assigned genotype is conditioned to facilitate the coding. This leads to a poor performance of  $K$  estimation as the likelihood



decreases and the variance of the likelihood increases considerably, making implausible K estimates (Pritchard *et al.* 2010).

Due to the fact that Structure was not able to detect any clustering pattern for the two *Astylosternus* species a better indicator of the population structure would be  $\Phi_{PT}$ . For *A. sp. 1* genetic differentiation via  $\Phi_{PT}$  was observed between the forest population MFIV and the riparian fragment populations F6 and KD4. As for *A. sp. 2* both  $\Phi_{PT}$  and Structure revealed no differentiation among populations. Likewise, more weight could be placed on the PCoA. It has been suggested that in polyploids multivariate analysis are suitable tools to infer evolutionary and genetic relationships between populations but also between individual genotypes (Dufresne *et al.* 2014). As stated by Jombart *et al.* (2009), multivariate methods are exploratory, thus among their requirements are not strong assumptions about an underlying genetic model, for instance the Hardy–Weinberg equilibrium and/or the absence of linkage disequilibrium, making them independent of ploidy level (Dufresne *et al.* 2014). Nevertheless, the PCoA presented here for *A. sp. 1* and *A. sp. 2* did not reveal a clear pattern between the individual genetic distances (Figures 4-11 and 4-14).

As stated by Slatkin (1985), the extent of gene flow among local populations determines their potential for genetic differentiation. Therefore, the lack of genetic differentiation among populations might be explained by current gene flow. The migration tests indicated that populations are moderately linked by gene flow. Current gene flow but also for the last two generations was detected for *Cardioglossa schioetzi* and *Leptodactylodon bicolor*. For the former, the test revealed that the population F5 contained immigrants from the other two riparian fragments but also from the forest population MFI. Then, individuals might be dispersing by following a stepping-stone dispersal model (Hedrick 2011) going for instance from MFI to F5 through F1 (see

Figure 4-1). Likewise, Robertson *et al.* (2008) indicated that tadpoles might be displaced likely due to periodic rises of stream water. Therefore, it could be said that the riparian forest fragments are acting as corridors promoting dispersal of individuals.

As for *Leptodactylodon bicolor*, the only riparian fragment F6 had immigrants from all the forest populations except from MFII; no particular pattern was observed for migration among the forest populations.

As revealed by the Mantel test, none of the four species exhibited a significant isolation by distance relationship. Geographic and genetic distances were not correlated significantly despite the considerable geographic distance between some of the populations. For instance, KD3 and MFIV are approximately 3.5 km apart. The lack of correlation suggested no significant restriction to gene flow among the populations due to distance over this fine geographic scale. Perhaps the gene flow and the relationship between geographical and genetic distances might have different patterns at larger spatial scales, that is, with farther distant populations an isolation by distance pattern may be detected. Unfortunately this statement cannot be supported as no information on the dispersal distances exists or has been published for any of the target species.

However, some examples are available for other amphibian species. For the salamander *Plethodon cinereus* Cabe *et al.* (2007) examined its genetic population structure at distances from 200m to 2 km, and found clear genetic differences among plots that increased with distance. While for the European tree frog *Hyla arborea* in a fragmented landscape, Angelone *et al.* (2011) reported that at distances shorter than two kilometers gene flow was affected only by physical barriers, however, at larger distances (from two to four km) gene flow was inhibited by geographic distance. A shortage of sample sites could be also the reason for the lack of correlation as the likelihood of detecting

isolation by distance increases with the number of populations sampled (Peterson and Denno 1998).

Based on population genetics theory, the lack of significant isolation by distance suggests that genetic drift and directional selection are playing an important role in population differentiation (Hedrick 2011), although in small populations, changes in allele frequencies are more strongly determined by genetic drift than by selection (Lacy 1987). For *Cardioglossa schioetzi* and *Leptodactylodon bicolor*, despite evidence of gene flow from the Structure analyses, significant genetic differentiation was detected between the forest and riparian forest fragment populations with  $\Phi_{PT}$ . Specifically, genetic divergence was observed between the forest MFI and the riparian fragments F1, F2 and F5 (see Table 4-4) for *C. schioetzi*, and for *L. bicolor* between the forest populations MFI and MFII and the riparian forest fragment population F6 (see Table 4-6). Thus, it is likely that genetic drift might be acting due to these riparian forest fragment populations being small and finite (Hedrick 2011). Gene flow mostly prevents genetic divergence between subpopulations, however, dispersal/migration may not prevent fragmented populations from losing genetic diversity more rapidly than single populations of the same total size (reviewed in Lacy and Lindenmayer 1995).

No previous studies have been reported on this matter for the focus species. This study represents the first attempt made to describe the genetic diversity and the population genetic structure of the target species of the Arthroleptidae family. Therefore, comparisons of the levels of genetic diversity and genetic structure found here to values in the published literature were not conducted.

#### 4.6.3 Genetic diversity and genetic population structure of polyploid species

During the field work one species of the *Astylosternus* genus was recorded. However, when preparing the herpetofaunal inventory (see chapter 2) the images of the animals and the field data results on the occurrence of amphibians revealed that actually two *Astylosternus* species occurred in the study area: *A. diadematus* and *A. rheophilus*.

Differences between adults of these two species are evident, however, the assignment of tadpoles to these species is uncertain because of their similarity. As most of the used tissue samples were from tadpoles it was necessary to assign species identity to the unknown specimens. Therefore, the named *A. sp. 1* and *A. sp. 2* were delimited by following the genotypic clusters criterion (e.g. Hausdorf and Hennig 2010) using AFLPs (Meudt *et al.* 2009). Nevertheless, for further studies on this species such difficulty needs to be solved. In order to assign species identity to each of the unknown specimens of *A. sp. 1* and *A. sp. 2* sequencing of mitochondrial gene fragments (e.g. 12S and 16S) were required, however by the time the project was conducted no more financial resources were available to complete such task.

As allopolyploidy has been confirmed for *Astylosternus diadematus* (Bogart and Tandy 1981), I decided to treat both *A. sp. 1* and *A. sp. 2* as polyploids. However, determination of ploidy should be confirmed by direct measurements (Bogart and Tandy 1976) because a number of the frog species recognized as polyploids have diploid populations. These authors conducted a comprehensive study of the chromosomes of African anurans and found that *Tomopterna delalandii* and *Hoplobatrachus occipitalis* have diploid and tetraploid populations. Thus, the assumption of polyploidy for *A. sp. 1* and *A. sp. 2* in this study is not necessarily correct.

It has been observed that in polyploids AFLP tend to produce higher numbers of fragments than diploids (Meudt and Clarke 2007); in addition the total number of fragments might increase as a result of homoplasy (comigration of nonhomologous fragments) for which AFLP have propensity (Caballero *et al.* 2008).

Contrary to what was expected, *Cardioglossa schioetzi* had higher levels of genetic diversity than *Astylosternus* sp. 1, however, it is important to note that the polyploid condition of the latter might contribute to or influence the levels of genetic variation. Despite the wide distribution and the high density of *A. sp. 1* in the study area, it had the lowest levels of genetic diversity (percentage of polymorphic loci as well as heterozygosity) among all other focus species. Many polyploidy organisms exhibit polysomic inheritance having higher levels of heterozygosity than their diploid progenitors (Soltis and Soltis 2000). Patterns of low genetic diversity have been reported in other polyploid species, mostly in plants, for which their genetic structure has been assessed by AFLP. For example, Kim *et al.* (2009) evaluated the genetic diversity and population structure of six *Isoetes* species from East Asia using AFLP, and observed that the levels of genetic diversity of two diploid species were higher than the tetraploid and hexaploid species. Furthermore, it should be noted that at the population level polyploids might have lower genetic diversity if polyploidy is a recent event (Glover and Abbott 1995). This is because the newly formed polyploids start with limited genetic diversity, get to the equilibrium between mutation and drift, and ultimately higher levels of genetic diversity would take a considerable amount of time to accrue (Glover and Abbott 1995, Arrigo and Barker 2012).

Nonetheless, that was not the case for *Astylosternus* sp. 2, for which the highest heterozygosity as well as the highest percentage of polymorphic loci for all the

populations of the four species were observed. Therefore, caution should be taken when the genetic diversity and the genetic population structure of polyploidy species are analyzed as interpretation of results might be erroneous. Most of the challenges associated with interpreting results on genetic diversity and genetic structure of polyploidy organisms arise because many of the standard tools for population genetics that have been developed for diploids, are not valid for polyploids (Dufresne *et al.* 2014). For instance, in the program Structure, Bayesian clustering based on HW equilibrium in polyploids might present several problems related mostly to violations of the basic assumption of random mating within clusters. As polyploids could shift to inbreeding and asexual reproduction, among other things, departures from Hardy Weinberg equilibrium might be observed representing a violation of the main assumptions of Structure (reviewed in Dufresne *et al.* 2014). As mentioned above for the two *Astylosternus* species, this would generate either a lack of population structuring or false population clustering (Pritchard *et al.* 2000, Pritchard *et al.* 2010).

#### **4.6.4 Implications for conservation**

For conserving and managing populations from a genetic approach, it is essential to have an understanding of the genetic processes that have shaped genetic variation and population structure (Schoville *et al.* 2011), especially for endangered populations. Information on the population structure of amphibian populations presented here will contribute towards effective conservation strategies and management decisions around Ngel Nyaki and Kurmin Danko forest reserve but also other montane forests in Nigeria and Cameroon.

Knowledge of dispersal in modified landscapes is limited for African amphibians (e.g. Measey *et al.* 2007, Sandberger *et al.* 2010). As a result, the generation of information which might provide a better understanding of dispersal in amphibians is crucial for determining the best strategy to mitigate negative effects of habitat fragmentation. Furthermore, information on dispersal patterns of the four target species in Ngel Nyaki and Kurmin Danko forest reserve can be extrapolated to other areas within the Mambilla Plateau.

Results from this study have provided valuable data on population genetic structure and differentiation among amphibian populations inhabiting disturbed riparian forest fragments but also continuous forest. Perhaps one of the most valuable pieces of information is the evidence of dispersal and gene flow. As revealed by the migration test, gene flow does exist between forest and riparian fragments, as shown for *Cardioglossa schioetzi* and *Leptodactylodon bicolor*. It was thought that the small frog *C. schioetzi*, inhabiting mostly riparian fragments severely impacted by human and cattle was going to be unable to disperse among other fragments or even to the forest. Nonetheless, the migration test indicated that current gene flow between the forest population and the three riparian fragment populations exists. From a conservation perspective the riparian fragments would be extremely important because they might be used as corridors for *C. schioetzi* and likely other species such as *Hyperolius riggenbachi* (see chapter 2) inhabiting mostly riparian habitats. Therefore, based on findings reported here, the maintenance of habitat connectivity should be a high priority for amphibian conservation in and around Ngel Nyaki and Kurmin Danko.

Immediate conservation actions to protect not only the target species but also the habitat of many other montane forest amphibians can now be taken. It is known now that

protection of riparian fragment is a priority in and around Ngel Nyaki and Kurmin Danko, however, on-going conservation actions (e.g. fences to prevent the cattle getting in) for the Ngel Nyaki forest should be reinforced for ensuring connectivity among forest populations. For amphibians connectivity is ultimately important even for populations of species that are not directly impacted by habitat loss or elevated mortality risks in dispersing (Cushman 2006).

It should be also noted that a remarkable contribution of this work was the rapid generation of molecular markers for the focal species. Simultaneous investigation of four threatened frog species was possible by applying AFLP, a low cost and a rapid technique that generated a high number of polymorphic loci that helped to assess the population genetic structure of such species in a relatively short period of time.

As stated by Richardson (2012), multiple species studies are critical to understand important differences in the response of distinct species to landscape modification that ultimately provide valuable information for the conservation management of an extensive area and diversity. In this particular study, protection of forests and riparian forest fragments can be extended not only to other areas within the Mambilla Plateau but also to the mountains in Cameroon where these species are distributed as well.



## **Chapter 5**

### **Discussion and future directions**

Conservation biology involves preserving species and their ecosystems by following three main approaches: 1) documenting the full range of biological diversity, 2) investigating human impact on species, genetic variation, and ecosystems, and 3) developing plans to prevent species extinction, maintain genetic diversity and protect biological communities and their associated ecosystem functions (Primack 1995). Such conservation approaches have been fulfilled through the development of the West African amphibian conservation project presented here. Firstly, the herpetofauna diversity on the Ngel Nyaki and Kurmin Danko Reserve was documented. Secondly, the genetic effects of montane forest fragmentation were investigated in four frog species. In order to assess the genetic diversity and the genetic population structure of these species the molecular markers AFLP were employed. Currently, amphibian conservation and forest management plans are being developed to protect the biodiversity on the Mambilla Plateau.

## 5.1 Base line data, the herpetofaunal inventory

A better understanding of the occurrence and number of species as well as their biology and ecology are invaluable for making conservation decisions (Vitt and Caldwell 2014). Following the first conservation biology objective, a herpetofaunal taxonomic survey was conducted in 2012, and a comprehensive inventory of the amphibian and reptile species in and around Ngel Nykai and Kurmin Danko was successfully compiled. Documenting the herpetofauna was crucial to prove occurrence, and to revise and update the distribution and conservation status of the amphibian and reptile species inhabiting such an important but unexplored area.

These surveys represent the most thorough inventory to date of amphibians and reptiles on the Mambilla Plateau. Previous surveys conducted in 1989 as part of a multidisciplinary expedition to eastern Nigeria recorded only two frog species (*Amietophrynus maculatus* and *Leptopelis nordequatorialis*) from the Ngel Nyaki Forest Reserve (Böhme and Nikolaus 1989). Then in 2009, during a rapid survey on Ngel Nyaki and Kurmin Danko forests, two frog species new to science were described; *Arthroleptis palava* (Blackburn *et al.* 2010) and *Phrynobatrachus danko* (Blackburn 2010). These four species were included in the inventory reported here.

An annotated list of 21 amphibians and 11 reptiles has been consolidated. Among the most interesting results were the new records for Nigeria and for the Mambilla Plateau. The frogs *Astylosternus* cf. *diadematus*, *A. rheophilus*, *Leptodactylodon bicolor*, *Hyperolius igbettensis*, and *Kassina decorata* were recorded for the first time in Nigeria. As for reptiles, it was documented here the first confirmed records of *Agama lebretoni*. Six other anuran species (*Arthroleptis* cf. *sylvaticus*, *Cardioglossa pulchra*, *C. schioetzi*, *Afrixalus quadrivittatus*, *Phrynobatrachus steindachneri*, and *Xenopus laevis*) and three

reptile species (*Rhinotyphlops decorosus*, *Cnemaspis spinicollis*, *Trachylepis* “sp. 4”) were reported for the first time on the Mambilla Plateau, thus underpinning the conservation of amphibians and reptiles in Nigeria’s montane forests. It should be stressed that these findings are of the utmost importance as the occurrence of some species in Ngel Nyaki and Kurmin Danko significantly increases the current knowledge of their distribution. This is true for the threatened frog species *C. pulchra* that was recorded only from Obudu Plateau and whose range is now known to extend into Taraba State of Nigeria. Likewise, these findings will undoubtedly help to fill a large gap in international knowledge around the distribution, species rarity, and evolutionary relationships of amphibians in this largely unexplored area of West Africa.

With regard to the conservation status of the amphibian species reported here, nine have been listed in the IUCN Red List (IUCN 2015) as Least Concern, five have been classified as Vulnerable and three have been listed as Endangered. One of which is *Cardioglossa schioetzi*, a target species of this project. According to the IUCN, this frog species has received such classification because of its fragmented distribution, its declining montane forest habitat in Nigeria, its population decline in Cameroon, and finally because its extent of occurrence is probably less than 5,000 km<sup>2</sup> (IUCN 2015). As for *Leptodactylodon bicolor*, *Astylosternus diadematus* and *A. rheophilus*, the loss of their forest habitat as well as their trend of decreasing population size have been significant to classify them as Vulnerable.

The inventory also helped to establish and/or verify the habitat status of some of the species. Chapman *et al.* (2004) evaluated the changes that the montane forest in Taraba state could suffer after their last description in the 1970s. They observed several abandoned farms within the limits of the forest reserve as well as soil erosion caused by

cattle movement. Outside of the reserve boundaries no legal protection has been provided to the riparian forests, and during the field work in 2012, severe damage to these sites by human activities was confirmed as the streams are used as watering holes and the constant crossing of cattle causes discontinuities along the fragments, ultimately promoting erosion and compaction of the soil. Among the riparian forests most negatively affected by cattle trampling were those where *Cardioglossa schioetzi* was found. The intensification of cattle grazing in this area (Hurault 1998) highlights the difficulties for conserving the forest and riparian forest fragments. This is of major concern because the persistence of populations in a fragmented landscape can be negatively affected by habitat size and quality (Fahrig 2003, Ye *et al.* 2013).

Besides the contribution to the hepatofaunal base-line data that will facilitate future additional studies of systematics and biogeography, this inventory is already contributing towards the on-going characterization of the biodiversity of the reserve, adding 32 more species to the growing list of the biodiversity of the Ngel Nyaki and Kurmin Danko forests, but also the Mambilla Plateau. Currently, the list of the amphibian and reptile species along with the biodiversity inventory of the Ngel Nyaki and Kurmin Danko can be consulted at the web page of the Nigerian Montane Forest Project at <http://www.afromontane.canterbury.ac.nz/biodiversity.shtml>.

## **5.2 AFLP, molecular markers used for the population genetics study**

Due to the increasing pressure generated by anthropogenic disturbances on the Mambilla Plateau, an immediate conservation action is required for preserving amphibian species. Consequently a rapid generation of genetic data is required. Nonetheless, there is no information on molecular markers used previously for the

target species. Microsatellites, the most commonly used markers for fine scale population structure studies for amphibians and reptiles (Squire and Newman 2002, Zamudio and Wieczorek 2007, Moore *et al.* 2008, Goldberg and Waits 2010) have not been yet isolated for any of the four target species. In fact, there are only two African amphibian species for which species-specific microsatellite markers have been isolated: the frog *Phrynobatrachus guineensis* (Sandberger *et al.* 2010) and the caecilian *Boulengerula* (cf.) *uluguruensis* (Barratt *et al.* 2012).

Due to the fact that the AFLP technique requires a short period of time to produce a large number of polymorphic loci (>1000) at relatively low cost (Bensch and Åkesson 2005), AFLP markers were employed for the population genetics study presented here. Through the assessment of two AFLP primer combinations this study demonstrated that AFLP markers are informative enough to be used for analysis at a population genetic level in the focal amphibian species for which no prior genetic information is available. This was supported with a high discrimination power obtained through the polymorphic information content (PIC); that is the markers' ability to differentiate between genotypes. The PIC was employed because in genetic studies it is among the most widely applied approach to measure the information content of molecular markers (Nagy *et al.* 2012) although other methods including the resolving power (RP) (Prevost and Wilkinson 1999) and the marker index (MI) are also used (Powell *et al.* 1996). Even though it is one of the most used approaches, no PIC values have been reported for any genetic study using AFLP in amphibians, so comparisons were only conducted based on published data in mammals and plants. The PIC results reported here (ranging from 0.211 to 0.283) were in agreement with those reported in cattle (0.08–0.38) (Ajmone-Marsan *et al.* 1997), but had more discrimination power than *Mucuna* (Fabaceae) (0.138 to 0.209) (Sathyanarayana *et al.* 2011).

The assessment of the AFLP markers (Chapter 3) indicated that the method was reproducible and although the genotyping error estimated for the two pairs of primers was slightly higher than the acceptable standard (Bonin *et al.* 2004), the markers were suitable genetic tools to assess the genetic diversity and genetic population structure for the four target species. It has been shown that errors in genotyping data have significant effects on parentage studies producing false paternity or maternity exclusion (Wang 2010). In the case of linkage and association studies genotyping errors might mask the true segregation of alleles (Pompanon *et al.* 2005). Nevertheless, in population genetic analyses (based on allele frequencies) the effect of error rates might be less severe (Pompanon *et al.* 2005, Dewoody *et al.* 2006).

Moreover, results confirmed the necessity to conduct informativeness assessment as well as reproducibility tests and genotyping error estimations (e.g. Crawford *et al.* 2012) as these approaches could be critical for applying markers of good quality that provide reproducible results and reliable estimations in genetic studies.

### **5.3 Advantages of AFLPS over other molecular markers for studies on genetic population structure**

The two preferred primer combinations ESP1B/MSP3 and ESP1B/MSP6 yielded 631 loci for the entire 396 samples, that is, the total number of samples including all four species. Out of the 631 loci, 554 (87.7%) were polymorphic. This level of polymorphism provided a high resolution for the detection of genetic diversity and genetic population structure of the four species although some results for two species (*Astylosternus* spp.) were to some extent restricted and cautiously interpreted (see below).

It has been recognized that for some genetic studies AFLPs are preferred over other molecular markers such as microsatellites. Comparing the utility of AFLPs *versus* microsatellites it has been demonstrated that for individual-based population assignment tests AFLPs performed better than microsatellites particularly when weak population structuring is detected (Campbell *et al.* 2003, Gaudeul *et al.* 2004). The reason for this is that a large number of loci are required for these kind of tests to get sufficient statistical power (Campbell *et al.* 2003). According to Gaudeul *et al.* (2004) the disparity between AFLPs and microsatellites is likely due to the capacity of AFLPs for screening many different DNA regions distributed randomly throughout the genome and the higher mutation rates of microsatellites. Gaudeul *et al.* (2004) also detected that at a small spatial scale AFLPs performed better than microsatellites. When using AFLPs, a higher differentiation of individuals from neighboring populations but also a higher percentage of individuals correctly assigned to their population of origin were observed than with microsatellites. Based on the information above, AFLPs were undoubtedly the most appropriate markers to be used in this study because no prior information was available for any of the target species, more than 500 polymorphic loci were rapidly obtained and ultimately appropriate for the geographic fine scale at which the population genetics study was conducted.

Some difficulties including the uncertain identification of the *Astylosternus* sp. 1 and A. sp. 2 and their polyploid condition were experienced in this study. While the use of AFLPs helped to partially solve the identification by delimiting these two species, it was impossible to get the maximum use or advantages of AFLP for these polyploid species. It should be noted there is a lack of population genetic studies with polyploid amphibians using AFLPs. This study emphasized the necessity of generating methods

and statistical tools adequate for polyploid organisms, especially for vertebrates in terms of population genetics.

During the field work one species of the *Astylosternus* genus was recorded; then when analyzing the images of the animals and the field data on the occurrence of the amphibian species it was revealed that actually two *Astylosternus* species occurred in the study area: *A. diadematus* and *A. rheophilus*. While the differences between adults of these two species could be easily detected, their tadpoles seem alike making it extremely difficult to distinguish individuals of one or another species. As most of the tissue samples were from tadpoles, it was necessary to assign species identity to the unknown specimens. Therefore, the named *A. sp. 1* and *A. sp. 2* were delimited by following the genotypic clusters criterion (e.g. Hausdorf and Hennig 2010). The delimitation of species boundaries was performed by using AFLPs (Meudt *et al.* 2009). Nevertheless, for further studies on this species such difficulty needs to be solved. It is paramount to assign species identity to the specimens of *A. sp. 1* and *A. sp. 2*, which ultimately will help to generate valuable information not only in terms of systematics, but also to develop further studies on genetics and biogeography.

Another difficulty was encountered with polyploidy of these *Astylosternus* species. At least for *A. diadematus*, allopolyploidy has been confirmed (Bogart and Tandy 1981), so it was decided that the two *Astylosternus* species assessed here should be treated as polyploids.

Using AFLP with polyploid species can have several advantages but also drawbacks. A very useful feature of AFLP in this study was the simultaneous generation of fingerprints for diploid (*Cardioglossa schioetzi* and *Leptodactylodon bicolor*) and polyploid species (*Astylosternus sp. 1* and *Astylosternus sp. 2*). Among the drawbacks



are the limited tools to evaluate the population genetics on polyploid species. Many of the approaches of population genetics have been designed for diploid organisms, thus cannot be valid for polyploids (Dufresne *et al.* 2014). This lack of validated methods could result in erroneous interpretations of the genetic structure results. It has been recognized that the Bayesian clustering would generate either a lack of population structuring or false population clustering in polyploids (Pritchard *et al.* 2000, Pritchard *et al.* 2010). Since the Bayesian clustering in the program Structure is based on Hardy-Weinberg equilibrium this may produce several problems in polyploids related mostly to violations of the basic assumption of random mating within clusters (Dufresne *et al.* 2014). As polyploids could shift to inbreeding and asexual reproduction (Mable *et al.* 2011), departures from HW equilibrium might be observed representing a violation of the main assumptions of Structure (Dufresne *et al.* 2014). Therefore, the population genetic structure reported here for the *Astyloternus* species was mainly based on the  $\Phi_{PT}$  measure of genetic differentiation.

Originally AFLPs have been widely used in plants (Bensch and Åkesson 2005), so it is not surprising that most of the studies of population genetic structure using AFLP are with polyploid plants (e.g. Burnier *et al.* 2009, Kim *et al.* 2009). In polyploid amphibians, studies have been conducted on *Xenopus (Silurana)* using AFLPs for genotyping sex to ultimately examine the effects of chemicals on gonad differentiation (Olmstead *et al.* 2010), generating linkage maps derived from joint segregation analyses of AFLPs (Kochan *et al.* 2003), and assessing hybridization using a modified methylation-sensitive AFLP technique (Koroma *et al.* 2011). Nonetheless, studies assessing the genetic structure of polyploid amphibians by AFLPs have not yet been published.

## 5.4 Genetic population structure

A better understanding of dispersal and connectivity among populations of the four focal species was acquired in this study. The analysis of AFLP to determine the population genetic structure revealed among other interesting results that continuous and fragmented populations are moderately linked by gene flow. The result of Bayesian estimates for recent migration rates indicated that current gene flow and for the last two generations was detected for *Cardioglossa schioetzi* and *Leptodactylodon bicolor*.

*Cardioglossa schioetzi* can move between the riparian fragments but also among the forest and the riparian fragments. For *L. bicolor* gene flow was also detected between the only riparian forest fragment and several forest populations. These findings identify the riparian fragments as a conservation priority on the Mambilla Plateau. In addition, this highlights the significance of corridors to facilitate amphibian dispersal, and the idea that these corridors can be used for other groups is not discarded (e.g. Burbrink *et al.* 1998, Vignieri 2005). Similar results have been reported for other anuran species using corridors for dispersal. For instance, in commercially managed forests, gene flow in the Rocky Mountain tailed frog *Ascaphus montanus* (Spear *et al.* 2010) and the Coastal tailed frog *Ascaphus truei* (Aguilar *et al.* 2013) took place along riparian corridors.

It has been suggested that when a large proportion of a landscape is of suitable habitat gene flow between populations is common, on the other hand, gene flow can be restricted if favorable habitat is not available (Spear *et al.* 2005). Likely the movement within the forest for *Leptodactylodon bicolor* is explained because of the presence of suitable habitat. Accordingly, it can be said that this species uses the riparian zones as corridors to disperse between the riparian forests but also between the forest and

riparian forest fragments, that is, these small riparian fragments are serving as stepping stones for species dispersal (e.g. Rubio and Saura 2012). As suggested by Forman (1995) the arrangement of fragments, corridors and matrix are critical for functional flows and movements of individuals through the landscape. For instance, empirical and simulation findings have indicated that for salamanders dispersal is facilitated by wide dispersal corridors (Kershenbaum *et al.* 2014). As for the target species more studies on linking landscape features to gene flow are required in Ngel Nyaki.

With regard to the movement within continuous habitat, there was no evidence that dispersal could be restricted for *Leptodactylodon bicolor*, *Astylosternus* sp. 1 and A. sp. 2. Although for the *Astylosternus* species the migration test was not conducted, this idea can be supported by the lack of genetic differentiation (based on  $\Phi_{PT}$  values) among populations.

None of the four species exhibited a significant isolation by distance relationship; geographic and genetic distances were not correlated significantly even though the geographic distance between some of the populations was large, for instance, KD3 in Kurmin Danko and MFIV in the Ngel Nyaki forest are approximately 3.5 km apart. This result is in the line with those reported for *Ambystoma maculatum*, in which no significant effect of geographical distance on patterns of dispersal was observed (Purrenhage *et al.* 2009).

To some extent this result is surprising because the opposite, that is, a significant isolation by distance relationship, has been reported for other amphibians (e.g. Vos *et al.* 2001, Spear *et al.* 2005, Angelone *et al.* 2011). It is possible that a shortage of sample sites could in part be responsible for the lack of geographic and genetic

distances correlation in this study, as the likelihood of detecting isolation by distance increases with the number of populations sampled (Peterson and Denno 1998).

Nevertheless, when putting all the outcomes together, the pattern of results presented here are similar to those found for *Phrynobatrachus guineensis* in Taï National Park (TNP), Ivory Coast (Sandberger *et al.* 2010). In contrast to what was expected, Sandberger *et al.* (2010) detected only a slight significant genetic differentiation among populations; likewise, no correlation between the geographic and genetic distances (isolation by distance) was observed, and the Bayesian clustering revealed no genetic substructure. Originally *P. guineensis* was thought to be weakly mobile and highly specialized, however high intra- and possibly inter-patch migration events explained the lack of population structure. Thus, individuals of this species are capable to disperse more than expected.

Likely some of the amphibian species inhabiting in and around the Ngel Nyaki forest could follow a metapopulation configuration (Hanski 1991). It has been observed that in fragmented landscapes some species persist as metapopulations (Hastings and Harrison 1994). Then, the persistence of genetic variation would depend on several factors such as high immigration rates and the ability of the species to form a metapopulation (Hanski and Gilpin 1997).

## **5.5 Importance of multispecies studies**

For conservation planning, studies assessing several species have the potential to complement ecosystem and single-species approaches (Schwenk and Donovan 2011).

As suggested by Richardson (2012), studies that take into account several distinct species are critical to detect different responses to the landscape modification. Different species might be affected in different ways by habitat degradation and fragmentation (Shreeve and Dennis 2011, Baguette *et al.* 2013, Carthew *et al.* 2013). Some species may face severe quick decline or simply disappear in habitat patches, others may remain relatively stable, or even can be benefitted by fragmentation (Laurance 2008). The spatial scale at which migration is no longer sufficient to preclude genetic structuring may therefore vary considerably between species depending on life-history and occupied habitat (Sandberger *et al.* 2010).

Documenting all these aspects is of utmost importance when planning management strategies of an extended area (e.g. Schwartz *et al.* 2007, Mortelliti 2013). The persistence of many species depends upon the effectiveness of strategies for conserving biodiversity in landscapes disturbed by human activities (Margules and Pressey 2000).

## **5.6 Conservation priorities and management plans**

Results from both the inventory and the genetic population structure study revealed that the riparian forest fragments are of utmost importance for the persistence and migration of *Cardioglossa schioetzi*, and potentially for many other amphibian species. By conserving forest stream habitats, the persistence of several species including the *Astylosternus* spp., *C. schioetzi*, and *Hyperolius riggenbachi* will be ensured. Although none of the anuran species reported here is restricted to forest, it is worth noting that *Leptodactylodon bicolor*, which was common along the streams within Ngel Nyaki forest, was recorded from only one riparian forest fragment outside of the reserve. Thus, efforts should be considered for protecting riparian zones but also for reinforcing

existing measures to protect the forest. The Nigerian Montane Forest Project (NMFP) has an on-going forest restoration project including the installation and maintenance of fences on the reserve perimeter allowing forest to regenerate but also preventing cattle going into the forest (Beck and Chapman 2008). These measures could indirectly help to protect the target and other amphibian species. Except for the species recorded within the boundaries of the forest reserve, which has been legally protected (Chapman and Chapman 2001), there has not been any conservation action involving the threatened target species on the Mambilla Plateau.

Previous studies on Ngel Nyaki revealed that the riparian forests are also important for other fauna groups such as invertebrates (e.g. Umar *et al.* 2013). Therefore, conservation and management plans should integrate results from all the biological groups already assessed in the study area as this would have broader biodiversity benefits. In this respect, the creation of buffer strips along the riparian streams is suggested. The retention of buffer strips along streams is among the most commonly recommended management strategies for reducing the loss of riparian zones as well as the impact of fragmentation on stream-dwelling amphibians (Reviewed in Curtis and Taylor 2003). Contributing to the regeneration of native riparian vegetation will provide a helpful buffer preventing erosion and sediment transport (Prosser *et al.* 2001). By conserving riparian fragments, not only can amphibians be protected, but also a number of invertebrates including crabs, dragon flies, beetles and many more insects that could be involved or be responsible for pollination.

Since the 1990s it has been pointed out that in spite of the high biodiversity value in West Africa a number of areas are endangered by habitat destruction, mainly by logging activities and human encroachment and no formal conservation status protection has

been provided (Stuart and Adams 1990). Nowadays the situation of many of these areas remains the same. Conservation efforts in many protected areas in West Africa remain minimal, additionally, important threats caused by human activities as well as population declines of wildlife still persist (Tranquilli *et al.* 2014). Thus, it is imperative to take immediate conservation action to protect biologically important areas such as the Mambilla Plateau in Nigeria.

Based on the results of this project, it is believed that multispecies studies are relevant to establish conservation priorities in the Mambilla Plateau. It is essential to produce as much information as possible on the abundance, life history, distribution, and genetics of several distinct species from this area. In addition, it is necessary to keep long-term monitoring programs not only to detect and mitigate population declines, but also to prevent potential extinctions on the herpetofauna in such an important area.

Research on the ecology and composition of the amphibian and reptile species will continue at Ngel Nyaki and Kurmin Danko forests to gradually add information to what is already known. Moreover, herpetofauna surveys will be extended to other montane forests in the Taraba State. New findings will substantially increase the knowledge of composition and distribution of the Central African herpetofauna and contribute towards effective conservation decisions.

The present study provides insight into the herpetofauna from the montane forest in the Mambilla Plateau, but other montane forests in Taraba State remain unexplored. This includes Chappal Waddi and Chappal Hendu within the Gashaka Gumti National Park for which field surveys are needed as almost nothing is known of the amphibian and reptile fauna in this region. Then, in order to complete the base line data on the herpetofauna of the Taraba State extensive and intensive taxonomic surveys are highly

recommended as part of an immediate conservation plan. Documenting the herpetofauna diversity will help to evaluate population trends, including population declines, as would conducting comparisons of the amphibian and reptile composition in other areas in Nigeria and comparisons to the montane forest biodiversity composition in Cameroon.

## **5.7 Significance of the new findings**

Findings from this work are now part of the valuable baseline data on the diversity and genetic population structure of amphibian species on the Ngel Nyaki and Kurmin Danko Forest Reserve on the Mambilla Plateau. The four target species have acted as a model to understand the genetic population structure of amphibians inhabiting disturbed areas due to anthropogenic activities such as agriculture, farming, and livestock on the Mambilla Plateau. The new scientific findings will better inform decisions to take immediate conservation actions as the amphibian species and their habitat are being dramatically affected by anthropogenic activities in the Mambilla Plateau. In addition, it has been observed that amphibians are sensitive to environmental health (Welsh and Ollivier 1998) making them an effective biological indicator not only in terms of habitat quality (e.g. DeGarady and Halbrook 2006) but also to detect population trends of other species (Beebee and Griffiths 2005).

By integrating results, the design and development of effective long-term conservation plans for Ngel Nyaki and Kurmin Danko Forest Reserve will be ensured. The conservation plans will include genetic and pathogens (specifically the detection of the fungus *Batrachochytrium dendrobatidis*) monitoring of endangered species, and the use of Geographic Information System (GIS) methodology to identify the potential



distribution of species. This strategy will provide measures to safeguard both forest and riparian forest habitats, and will also facilitate the formation of partnerships to sustain research programs not only in Nigeria but also in other highlands in Cameroon.

An important outcome was the impact the project had on people working and living in and around Ngel Nyaki forest. During the field work villages were aware of the importance of amphibians, and by the end of it there was a change from negative or indifferent attitudes towards amphibians into positive ones. This project was part of the Nigerian Montane Forest Project, as such it contributed towards employment and training of members of the local communities. Likewise, through participation in this project, Nigerian undergraduate students were exposed to amphibian ecology and conservation. This will undoubtedly have a long term impact on those who continue in the field of conservation and those who will make future decisions around land use.

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# Appendix I

## CTAB method of DNA extraction (amphibian tissue)

### Modified from (Weising *et al.* 1995)

(CTAB or Cetyltrimethylammonium bromide is also known as Hexadecyltrimethylammonium bromide).

CTAB buffer:

0.1M Tris pH 8.0, 1.4M NaCl, 20mM EDTA pH 8.0 and 2% CTAB.

- 1) Place sample in 1.5ml tube (3 to 5 mm<sup>2</sup> approximately) with 500µl 2xCTAB buffer (0.1M Tris pH 8.0, 1.4M NaCl, 20mM EDTA pH 8.0, and 2% CTAB), add 5 µl proteinase K (20mg/ml).
- 2) Incubate in heat block at 56°C for 4 hours (or overnight), with occasional mixing.
- 3) Cool tubes for a few minutes then add 600µl chloroform-isoamyl (24:1). Vortex shortly, mixing the whole contents of the tube.
- 4) Centrifuge 10 minutes at maximum speed (13000 rpm). With a pipette transfer the supernatant into a new 1.5ml tube.

- 5) Add 600µl chloroform-isoamyl (24:1) to supernatant. (Chloroform-isoamyl alcohol is toxic upon inhaling and should be handled in a fume hood). Vortex shortly.
- 6) Centrifuge 10 minutes. Pipette supernatant into new 1.5ml tube.
- 7) Add 4µl RNase (10mg/ml) and incubate 30 minutes at 37°C.
- 8) Add 0.7 volumes of cold isopropanol. Invert several times. Put tubes in freezer (-20°C) overnight.
- 9) Centrifuge at maximum speed for 10 minutes.
- 10) Discard the supernatant. Wash the DNA pellet with 300µl 95% ethanol.
- 11) Centrifuge at maximum speed for 10 minutes.
- 12) Discard the supernatant. Wash the DNA pellet with 300µl 70% ethanol
- 13) Centrifuge 10 minutes, discard the supernatant.
- 14) Dry the pellet by leaving tube with lid open for about 15 minutes.
- 15) Dissolve the DNA pellet in TE buffer (30-100 µl). Leave overnight in fridge to allow time to dissolve or heat at 65°C in heat block for 5 – 20 min to speed up the dissolving process.
- 16) Estimate DNA quality/quantity on a 1% TBE agarose gel or using a spectrophotometer (NanoDrop ND-1000).
- 17) Store DNA extracts at -20°C or -80°C (recommended for a long-term storage).

## **Appendix II**

### **General framework for Laboratory Work:**

DNA extractions should be visualized on a 1% agarose gel to ensure their quality and quantity. It has been demonstrated that measuring the DNA concentration using a spectrophotometer is not enough because DNA could be degraded despite its concentration.

Chloroform, isoamyl alcohol used on the CTAB extraction protocol is toxic upon inhalation, thus for safety precautions it should be handled in a fume hood.

DNA digestion, ligation and pre-selective PCR amplification can be completed in one or two days (depending on the preferred incubation period of ligation reactions). This prevents a few technical difficulties such as star activity and degradation of enzymes due to not ideal storage conditions (e.g. overnight storage conditions at 4°C will produce star activity of restriction enzymes or degradation of ligation reaction).